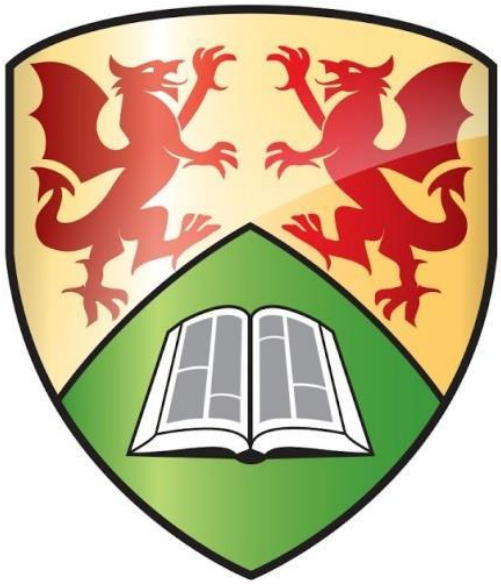
Molecular approaches to uncover the fundamental biology of *Calicophoron daubneyi*

by

Nathan Rhys Allen



A thesis submitted at the Institute of Biological, Environmental and Rural Sciences (Aberystwyth University), for the degree of Doctor of Philosophy

November 2019

DECLARATION

WORD COUNT OF THESIS: 46,278

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ..... (candidate)
Date .....

STATEMENT 1
This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references.
A bibliography is appended.

Signed ..... (candidate)
Date .....

STATEMENT 2
I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ..... (candidate)
Date .....

SUMMARY

Over the past decade rumen fluke have emerged as a major parasite of livestock in Western Europe. Although recognised to cause clinical and sub-clinical disease in non-temperate regions, there have only been few studies into the potential economic losses associated with infections in temperate areas. Given a status as a newly emerging parasite, many aspects of the fundamental biology of potentially the most dominant temperate rumen fluke, *Calicophoron daubneyi* have yet to be researched in detail.

In the current thesis, many characteristics of *C. daubneyi* basic biology have been uncovered using a combined proteomic and bioinformatics approach that have produced an array of datasets that will aid future applied studies. The global soluble somatic and egg proteomes of *C. daubneyi* were successfully resolved utilising SDS-PAGE combined with LC-MS/MS elucidating an array of protein families including fatty acid binding proteins (FABPs) and alpha crystallin containing small heat shock proteins (AC/sHSPs) with the potential to be used as biomarkers in infection diagnostics due to their immunogenicity. Extracellular vesicle (EV) isolation techniques previously utilised in parasitic flatworms were exploited facilitating the successful isolation of EVs from *C. daubneyi* ES products for the first time. Furthermore, proteomic investigation identified a multitude of proteins such as Sigma-class GST and cathepsins L and B in EVs that have previously been described in immune evasion and successful establishment of helminth parasites allowing insights into the mechanisms of establishment utilised by *C. daubneyi*. Further investigation into isolated EVs highlighted their antimicrobial activity as well as mechanisms of EV release, all of which can potentially be utilised in the future treatment of infection. All proteomic profiles resolved contained numerous hypothetical and uncharacterised proteins that are likely specific to *C. daubneyi* and their further study could be key to understanding the mechanisms through which the parasite establishes successfully and elucidate treatment options. Currently, there are no licenced anthelmintic treatment options to *C. daubneyi* in the UK and with its prevalence increasing it is likely that alternative treatment options will be required in the near future in order to combat the likely economic impact of increased infections. With increasing prevalence, it is of great importance to understand the capacity and mechanisms of xenobiotic detoxification in *C. daubneyi* in order to allow the development of effective anthelmintics. Phase I and II detoxification enzymes, glutathione transferase (GST), sulfotransferase, monoamine oxidase and cytochrome P450 were identified in the *C. daubneyi* transcriptome, providing evidence of drug metabolism capacity that could support future resistance of an anthelmintic. However, Phase I and II protein presence was also investigated in the resolved proteomes, with only Phase II GSTs identified during i*n vitro* culture highlighting their importance in *C. daubneyi* successful establishment and maintenance of infection.

With its status as a rapid newly emerging parasite in the UK, it is likely that *C. daubneyi* will continue to increase in prevalence across the UK and Europe requiring a need for the development of treatment options with many aspects of *C. daubneyi* still requiring research in order to decrease the potential threat to livestock production that is likely to be observed. The results from this research have uncovered many aspects of *C. daubneyi* fundamental biology, including highlighting specific protein families of interest that may prove useful as diagnostic markers and potential vaccine and anthelmintic candidates.

TABLE OF CONTENTS

ABBREVIATIONS

ºC	Degrees Celcius
1-DE	One dimensional electrophoresis
2-DE	Two dimensional electrophoresis
AC/sHSP	Alpha crystallin containing small heat shock protein
ACN	Acetonitrile
AMBIC	Ammonium Bicarbonate
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CDNB	1-chloro-2,4-dinitrobenzene
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CYP	Cytochrome p450
DC	Differential centrifugation
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
ESCRT	Endosomal Sorting Complex Required for Transport
ESI	Electrospray ionization
ESP	Excretory/secretory products
EST	Expressed sequence tag
EV	Extracellular vesicles
FABP	Fatty acid binding protein
FEC	Faecal egg count

FMO	Flavin containing monooxygenase
GO	Gene ontology
GSH	Glutathione (g-glutamyl-cysteinyl-glycine)
GST	Glutathione transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA	Iodoacetamide
IEF	Isoelectric focussing
ILV	Intraluminal vesicle
IPG	Immobilised pH gradient
kDa	kilo Dalton
MAO	Monoamine oxidase
ml	Millilitre
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
MVB	Multivesicular body
MW	Molecular weight
MWCO	Molecular weight cut off
NCBI	National Centre for Biotechnology Information
PAGE	Polycrylamide gel electrophoresis
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
Q-TOF	Quadrupole time of flight
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC	Size exclusion chromotography
SULT	Sulfotransferase
tBLASTn	Translate blast nucleotide
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TGS	Tris glycine SDS
UGT	UDP-glucuronosyltransferase
UK	United Kingdom
v/v	Volume/volume
w/v	Weight/volume
WHO	World health organisation
XME	Xenobiotic metabolizing enzymes
µg	Microgram
µl	Microlitre

CHAPTER 1.

GENERAL INTRODUCTION

1.1 PARAMPHISTOME EPIDEMIOLOGY

1.1.1 *Introduction*

Livestock infections with parasitic helminths are a major problem in agriculture worldwide, often representing the primary cause of productivity loss in the industry due to the implications of clinical and subclinical losses, as well as the costs associated with the implementation of control and treatment strategies (Ballweber, 2006; Donald, 1994; Waller, 2003). Currently, there are only a few viable options for controlling parasitic helminths infecting livestock, including biological control and pasture management, with treatment using anthelmintic drugs remaining the primary method of management in the continued absence of vaccines (Mitreva *et al.,* 2007).

In regard to treatment of helminth infections, the British Veterinary Association (BVA) guidelines state: 'Ensure full grazing management programmes and the use of regular faecal egg counts (FEC) to ensure treatment of only those animals that need it' (Forbes, 2017). Minimisation of productivity losses can be achieved through early identification of infection and subsequent treatment, with strategic plans endeavouring to achieve avoidance of parasite exposure advantageous (Forbes, 2019). Many parasitic helminths have specific definitive hosts which can be monopolised in their control and treatment through mixed or co-grazing, however many parasites including fluke species have multiple potential mammalian hosts and so the variances in host responses can be exploited to ensure their successful management (Forbes, 2017). Due to the importance of livestock farming on the future of food security, it is of upmost importance to ensure there are effective mechanisms of management of parasitic helminth infections to ensure efficiency in farming and its sustainability (Charlier *et al.,* 2015).

1.1.2 ***The Trematoda***

Parasitic helminths are of great veterinary, agricultural and medical importance as the family responsible for a wide selection of disease (Cuesta-Astroz *et al.,* 2017). In agriculture, helminths are responsible for substantial losses with billions of dollars spent annually on treatment and control (Robinson & Dalton, 2009; Wang *et al.,* 2009; Perry & Randolph, 1999). As a family, helminths are divided into two phyla, the Nematodes and Platyhelminths (Hotez *et al,* 2009). A majority of parasitic helminths belong to the roundworms (Phylum Nematoda) and the two classes within the Platyhelminthes, the flukes (Trematoda) and the tapeworms (Cestoda) (Franchini *et al.,* 2015), with the World Health Organisation (WHO) detailing 8 out of the 17 defined neglected tropical diseases to be caused by helminth parasites. The trematoda as a class is divided into two defined subclasses, the Aspidogastrea and the Digenea. The Aspidogastrea represents the smaller of the two (~80 species) with low economic importance with infections primarily affecting molluscs and vertebrates, whilst the Digenea is a significantly larger subclass (~18,000 species) with a vast number of genera and a diverse range of host species (Olsen *et al.,* 2003; Konstadinova & Pérez-del-Olmo, 2019). The taxonomy of the Digenea has been complex to define due to issues in identifying relationships and methods of diagnosis, with morphology of all Digenean trematodes found to be visually analogous with dorsoventrally flattened leaf shaped bodies ranging from several millimetres up to 8cm in length (**Figure 1.1**). Historically over 2500 genera had been categorised into the digenea, however recent molecular studies have only confirmed 148 families comprised of 1577 genera (Toledo & Fried, 2017).

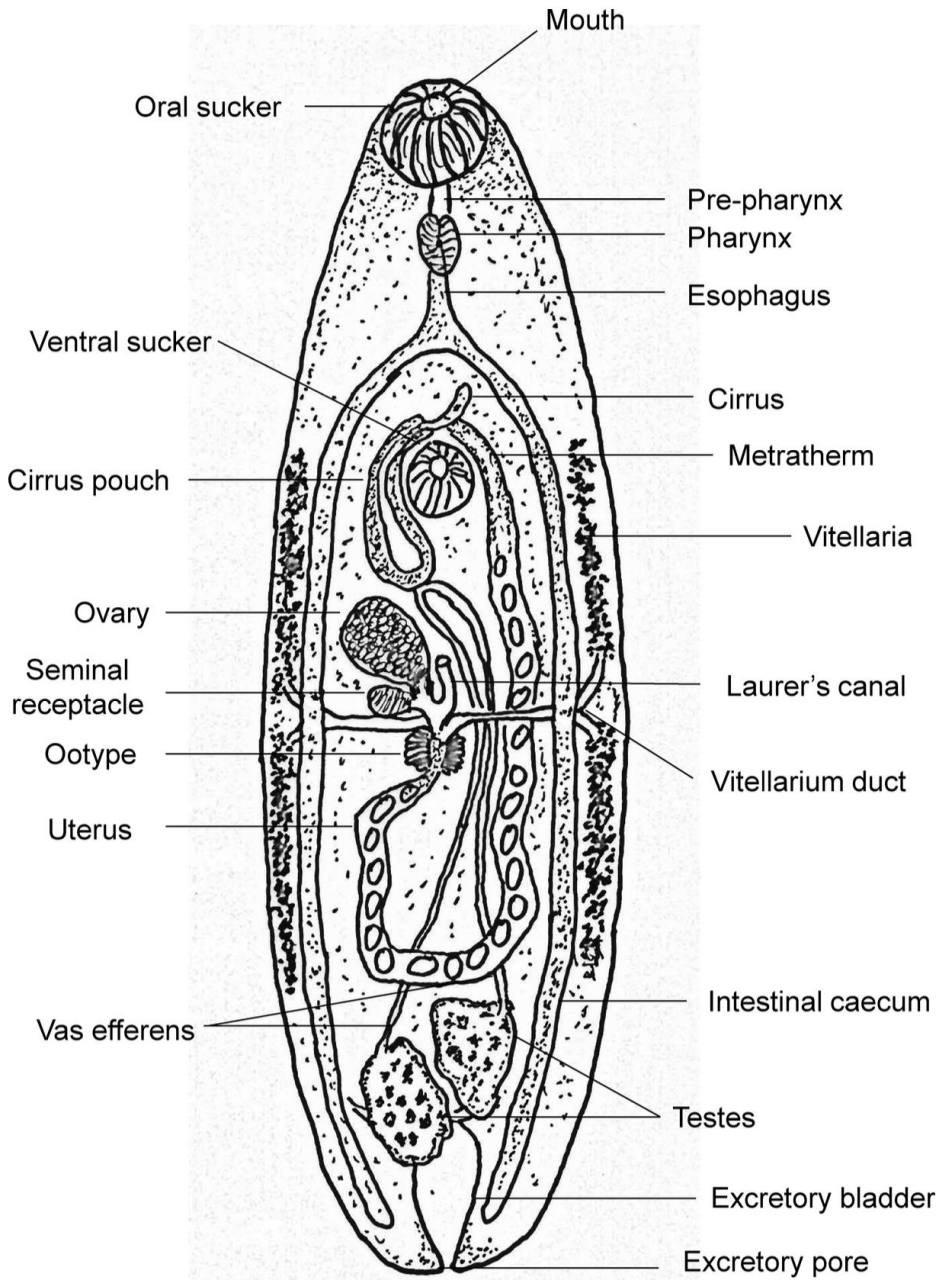


Figure 1.1: Generic organography of an adult digenic trematode (Toledo & Fried, 2017).

1.1.3 ***Paramphistomes***

Tissue and blood trematodes can be detrimental to livestock producers due to their contribution to morbidity and mortality as well as associated sub-clinical disease leading to loss of economic returns. Thus, the trematodes contains species found in the liver, gastro-intestinal tract or blood vessels of their hosts (Rojo-Vázquez *et al.,* 2012), with tissue trematode parasites found to reside in the GI tract largely understudied due to the prevalence, distribution and morbidity associated with liver and lung flukes (Sen-Hai & Mott, 1994). Specifically, the trematode parasites within the family paramphistomidiae collectively make up the group known as paramphistomes - more commonly referred to as the rumen or stomach flukes. Ruminant infections with paramphistomes have been observed worldwide and represent the predominant parasite found in both the rumen and reticulum of sheep, goats, cattle and water buffalo (Anuracpreeda *et al.,* 2008). Paramphistomes can be divided into numerous genera including *Paramphistomum, Calicophoron, Cotylophoron* and *Balanorchis,* all of which have been identified as infecting cattle in a diverse range of geographical locations (Tandon *et al.,* 2014). As a clade, paramphistomes encompass over 20 species, however it has been noted that *Calicophoron daubneyi* (*C. daubneyi*) is the only species to infect livestock in Western Europe (Soulsby, 1965; Jones *et al.,* 2015). Despite their worldwide prevalence, some fundamental information about paramphistomes is still unknown, including many aspects of their fundamental biology, methods of diagnosis and potential treatment and control options (Huson *et al.,* 2017).

1.1.4 ***Paramphistomosis***

Paramphistomosis is the clinical disease caused by infection with trematodes belonging to the paramphistomidae family and is generally the result of a largely

neglected, high burden infections (Anuracpreeda *et al.,* 2008; Rojo-Vázquez *et al.,* 2012). Paramphistomosis is responsible for drastic economic losses in a range of tropical and sub-tropical regions, however it has now been found to affect ruminants worldwide (Rojo-Vázquez *et al.,* 2012; Diaz *et al.,* 2006). In recent years Great Britain and Ireland have seen substantial increases in the number of paramphistome eggs in cattle faeces leading to increased interest into the research of the parasites responsible for the infections (Foster *et al.,* 2008, Murphy *et al.,* 2008). Clinical symptoms including morbidity of infection has been attributed to sexually immature worms in their migratory stage rather than adult fluke in their definitive position within the host and can be potentially fatal in sheep and cattle (Horak, 1971; Forbes, 2018). Clinical symptoms present following excystment and migration of immature fluke to the duodenum mucosa where they develop and subsequently penetrate the mucosal lining causing significant damage to the tissue with high burdens leading to propagation of acute inflammation and pathology (Millar *et al.,* 2012; Mason *et al.,* 2012; Tilling, 2013). In extremely high numbers, immature fluke can cause fatality through widespread damage of intestinal tissue leading to haemorrhaging particularly in juvenile animals (Mason *et al.,* 2012). Interestingly fatalities have only been identified in calves yet have been identified in sheep of all ages (Millar *et al.,* 2012; Forbes, 2018). Adult fluke have not been found to cause mortality, however, ruminal papillae atrophy and rumen wall ulceration at the site of attachment has been observed, which have been associated with decreased milk yields and growth rate (Foster *et al.,* 2008; Fuertes *et al.,* 2015). Paramphistomes have been found to mature and migrate rapidly in cattle, when compared to other hosts, as well as living and producing eggs for a longer period of time (Horak, 1971). Clinical paramphistomosis has mainly been

observed between September and December which is similar to many clinical trematode infections, including *Fasciolosis* (Alzieu & Dorchies, 2007).

1.1.5 ***Calicophoron daubneyi***

Calicophoron daubneyi (*C. daubneyi*) is a gastric trematode with particular pathology in young ruminants. It was first classified by Eduardo (1983) after revision of the genus *Calicophoron* following its initial characterisation as *Paramphistomum daubneyi* by Dinnik (1962) (Diaz *et al.,* 2006). C. *daubneyi* was first confirmed as being present in the UK in 2012 (Gordon *et al.,* 2012), with prevalence now found to be high with abattoir studies reporting 29% of cattle in the UK to be infected (Saringson *et al.,* 2016). Adult *C. daubneyi* have been shown to induce an inflammatory response in the rumen and reticulum in which they reside (Fuertes *et al.,* 2015), whilst juvenile stages of paramphistomidae are known to cause pathology and even fatality in large numbers due to the inflammatory immune response incited by their attachment to the small intestine wall (Singh *et al.,* 1984). This attachment can also result in damage to the mucosal membrane leading to ineffective digestion and absorbance through the intestinal wall, causing enteritis, diarrhoea, anaemia and weight loss (Spence *et al.,* 1996). The main effect on adult ruminants is a decrease in food conversion resulting in weight loss and decrease in milk production causing large economic losses (Rangel-Ruiz *et al*., 2003). Early reports of rumen fluke in the UK were based solely on morphological identification, identifying *Paramphistomum cervi* as the main species affecting British livestock (Pillers, 1922). However, recent molecular studies have all identified *C. daubneyi* as the species infecting UK livestock (Gordon *et al.,* 2013; Huson *et al.,* 2015).

1.1.6 ***Morphology***

Paramphistome infections are identified based on their morphology, carried out through their size, shape and presence of anterior and posterior suckers. Immature paramphistomes are less than 1 mm in length and reside in the small intestine of the host, with their presence observed as little as 9 days following exposure. Small adult fluke appear bright red in colour around 2-3 mm in length, whilst adult paramphistomes are typically 0.5-1.0 cm long and found to be firmly attached to the rumen wall by their acetabulum with their surface covered in papillae (**Figure 1.2**) (Huson *et al.*, 2017; Eduardo, 1983). Paramphistome eggs are colourless with an approximate length of 130-180 µm (Taylor, 2007). Eggs of both *C. daubneyi* and closely related trematode *F. hepatica* are morphologically similar, with *C. daubneyi* eggs appearing clear whilst *F. hepatica* are yellow (Foster *et al.*, 2008).

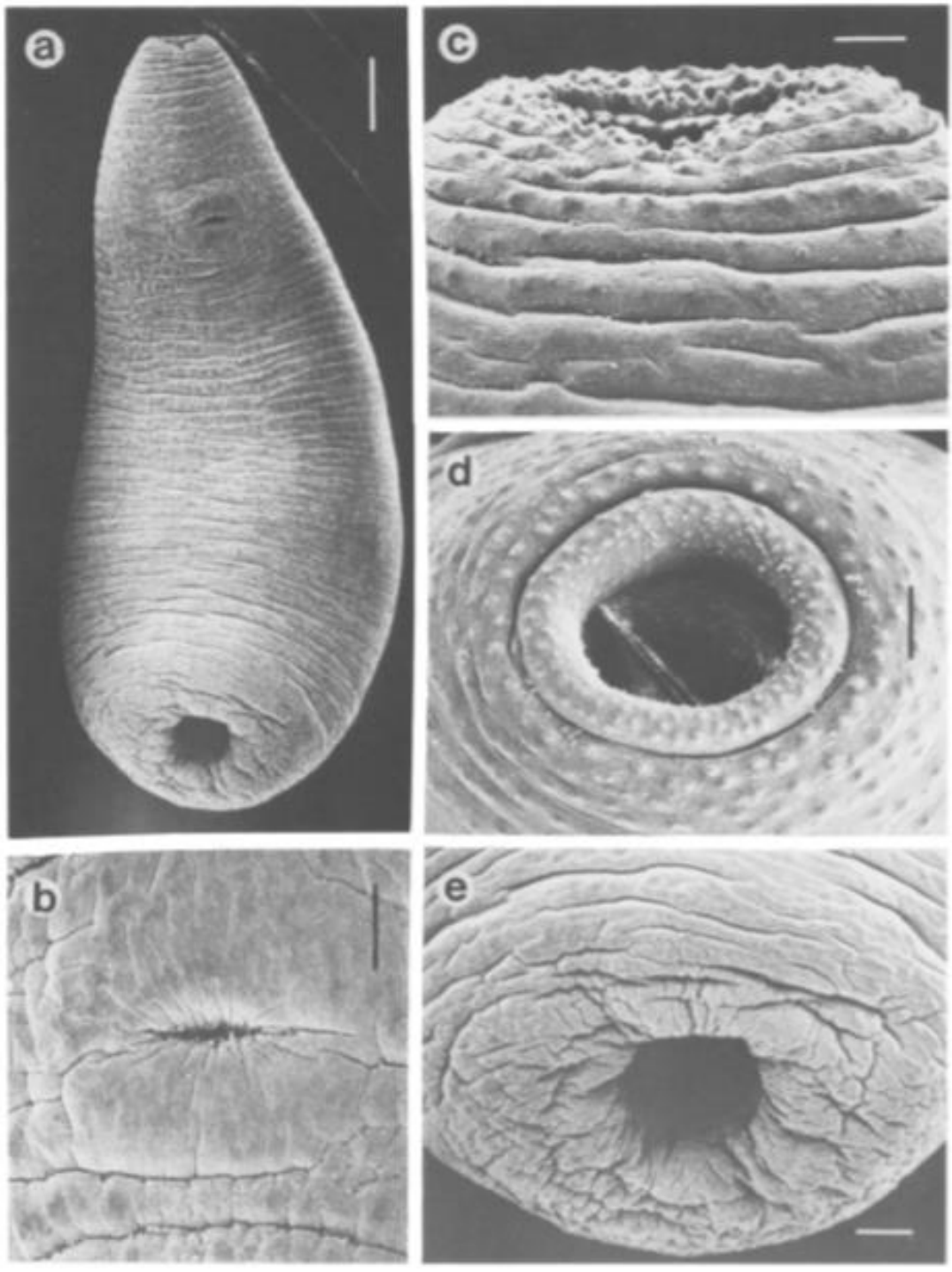


Figure 1.2: SEM images of *C. daubneyi* from Dinnik, 1962. a. Whole worm, ventral view (scale 500 µm) b. Genital pore (scale 100 µm). c. Anterior end (scale 100 µm). d. Anterior end (scale 100 µm). e. Acetabular region (200 µm). Images remastered from Eduardo (1983).

1.1.7 ***Emerging livestock parasite***

Increasing parasitism of livestock worldwide has been attributed to two main factors – development of anthelmintic resistance and demand for organic farming (Waller, 2003). Although long regarded as only common in non-temperate regions, cases of paramphistomosis have been reported to be increasing in temperate zones across South America and Europe, including the UK and Ireland (Sanabria & Romero, 2008; Malrait *et al.,* 2015; Jones *et al.,* 2017; Martinez-Ibeas *et al.,* 2016). Whilst trematode infections such as fasciolosis are well documented and known for its vast economic loss to the farming industry, it is reported that the economic impact of paramphistomosis are largely underestimated (Mage *et al*., 2002).

Paramphistome infections have been identified in ruminant livestock in the UK for over a century, with studies morphologically identifying the species to be *Paramphistomum cervi (P. cervi*) infecting both sheep and cattle (Sey, 1980; Piller, 1922). Historically rumen fluke infections were only reported occasionally in the UK and Ireland, with many early studies reporting low prevalence rates (Kelly, 1948; Willmott, 1950). Studies over the past 20 years have shown an increase in cases of infection across Europe with levels of 16-77% recorded on farms (**Table 1.1**). However, it is difficult to make comparisons between studies due to differences in livestock species and methods through which sampling took place. It is also difficult to determine the species of rumen fluke during collection due to their morphological appearance of both themselves and the eggs they produce (Choudhary *et al.,* 2015). Eggs produced by the parasite have only been identified in faecal samples within the UK since the mid-2000s, affirming its status as a newly emerging parasite of domestic livestock with infections now commonplace across the UK and Ireland (Sargison *et*

al., 2019). Despite its importance and possible future detrimental effects on the livestock industry its basic biology and interaction with the host are still poorly understood and studied (Huson *et al.,* 2018).

Increased incidence of rumen fluke infections across Western Europe have been attributed to the single paramphistome species – *C. daubneyi*, with prevalence continuing to increase to date (Toolan *et al.,* 2015). The reasons behind initial emergence and increasing prevalence of *C. daubneyi* infections in the UK are still poorly understood despite their possible impact on production loss due to their documented causation of clinical disease in subtropical regions (Sargison *et al.,* 2019; Rangel-Ruiz., 2003). Emergence of *C. daubneyi* and subsequent spread of infection could be attributed to many factors including the change in climate conditions permitting optimal environments for completion of the life cycle as well as increased availability of *G. trunculata* as its intermediate host due to regular *F. hepatica* treatment (Jones *et al.,* 2017; Skuce *et al.,* 2013).

Table 1.1 Prevalence of Rumen Fluke parasites in European countries (Jones, PhD Thesis, 2017).

Year	Country	Method	Animal	N	Prev. %	Study ref.
1999	FRA	Vet. Surv.	Cattle	1310	45	Mage et al. (2002)
2000-01	ITA	Farm (FEC)	Sheep	197[a]	16	Cringoli et al. (2004)
2001-04	ESP	Farm (FEC)	Cattle	121 [a]	36	Díaz et al. (2007)
2006	FRA	Farm (FEC)	Goats	42[a]	58	Paraud et al. (2009a)
2007-09	ESP	Abattoir	Cattle	775	36	Arias et al. (2011)
2008	ESP	Abattoir	Cattle	589	19	Gonzalez-Warleta et al. (2013)
2013	BEL	Abattoir	Cattle	125	28	Malrait et al. (2015)
2013-14	IRE	Abattoir	Cattle	518	52	Toolan et al. (2015)
2013-14	IRE	Abattoir	Sheep	158	14	Toolan et al. (2015)
2014	UK	Abattoir	Cattle	974	25	(Bellet et al., 2016)
2014	IRE	Farm (FEC)	Sheep	304[a]	77	(Martinez-Ibeas et al., 2016)
2014	UK	Abattoir	Cattle	339	29	(Sargison et al., 2016)
2014	NET	Abattoir	Cattle	116	23	(Ploeger et al., 2017)

[a] flocks/herds.

1.1.8 ***Life cycle***

Digenean lifecycles are complex, developing through a number of stages in the external environment before entering their intermediate and definitive host (**Figure 1.1**). Paramphistomes have a heteroxenous life cycle with fresh water snails acting as their intermediate hosts (Malrait *et al.,* 2015). *C. daubneyi* and *F. hepatica* have similar life cycles with analogous periods of infection risk due to their shared intermediate host, *G. trunculata* (Dreyfuss, 2015), with the possibility of this molluscan intermediate host being dually infected (Augot *et al.,* 1996). Paramphistomes have a two-host life cycle involving an intermediate aquatic molluscan host and a definitive vertebrate host.

Successful establishment and maturation within the definitive host leads to the production of eggs that are secreted in the faeces where they hatch before finding an appropriate intermediate host to continue the cycle (Zintl *et al.,* 2014). Within the intermediate host, paramphistome miracidium go through three lifecycle stages as sporocysts, rediae and cercaria. Following maturation as cercariae, they are released onto vegetation where they encyst to metacercariae where under favourable conditions they can remain dormant for long periods until they are ingested by their definitive hosts (Huson *et al.,* 2017; Sanabria & Romeo, 2008). Upon ingestions, metacercaria encyst in the duodenum of the small intestine where they are thought to plug attach allowing them to feed and develop prior to migration along the intestinal tract through the duodenum, abomasum and omasum until they reach the rumen and attach to the rumen wall where they mature into adults (Sanabria & Romero, 2008; Zintl *et al.,* 2014). Upon maturation the fluke produces eggs that are then released in the faeces allowing the lifecycle to continue.

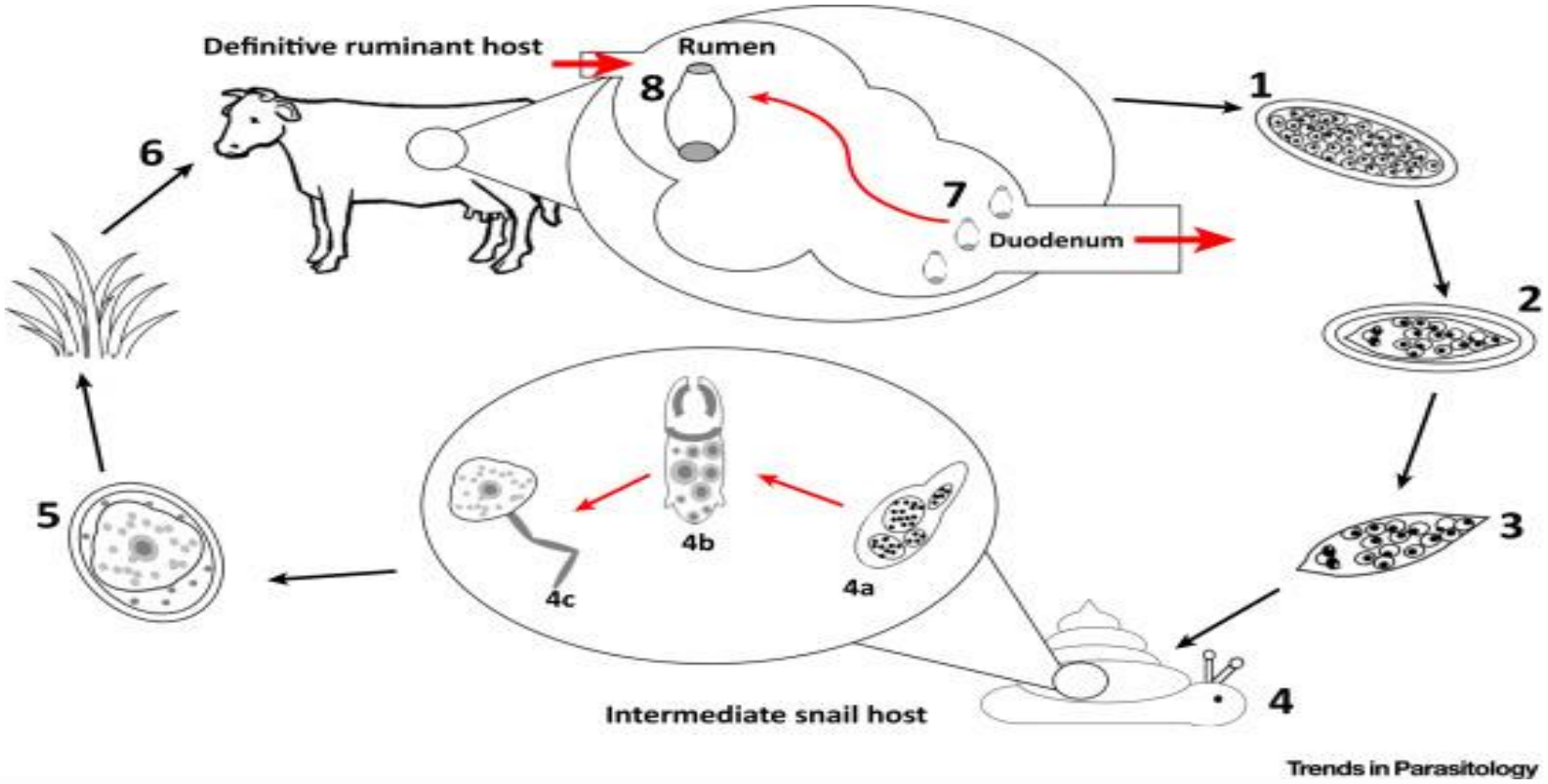


Figure 1.3: Life-cycle of *Calicophoron daubneyi*. Eggs produced in the Rumen are passed through the GI tract and into the environment in the definitive hosts faeces (1). Under optimal conditions eggs embryonate (2) and hatch as miracidia (3). They locate an intermediate host (e.g. *Galba trunculata*) (4), in which they asexually reproduce and go through three larval stages, sporocysts (4a), rediae (4b) and cercariae (4c). Following this maturation free-living cercariae are released onto vegetation and undergo encystment. These metacercariae remain dormant until they are consumed by the definitive ruminant host (6). They then encyst in the duodenum of the small intestine (7) before migration into the Rumen when fully mature where they attach to the rumen wall/papillae using their muscular acetabulum (Huson *et al*, 2017).

1.1.9 ***Diagnosis of infection***

There is currently no validated diagnostic method through which clinical cases of paramphistomosis caused by intestinal residing juveniles can be identified, with infection often only noted following post-mortem examination (Huson *et al.,* 2017). The current method of identifying adult paramphistome infections is through faecal egg counting (FEC), utilising either flotation or sedimentation of eggs (Gordon *et al.,* 2012). However, there are many limitations to FEC, one of which is the similarities in eggs between *C. daubneyi* and *F. hepatica* that may lead to false positives in the diagnosis of infection (Gordon *et al.,* 2013; Rojo-Vázquez *et al.,* 2012). False diagnosis can lead to downstream complications due to treatments for *F. hepatica* (excluding oxyclozanide) remaining ineffective against paramphistome *spp*. (Rolfe & Boray, 1987), leading to eggs remaining in faeces that can lead to unwarranted further treatment that will be cost ineffective to livestock farmers. FEC also has drawback in the time it takes for the parasite to establish and begin releasing eggs from the host, this is also the period in which the infection has the potential to cause mortality in the host without being able to be detected. A further method of diagnosis is through use of ELISA utilising proteins specific to paramphistome species, however, currently used ELISAs have been acknowledged as having cross-reactivity with closely related trematode species such as *F. hepatica* (Díaz *et al*., 2006). Increased prevalence of rumen fluke infections have been identified using two main methods of identifying infections, FEC and examination of the animals at slaughter, with the latter also allows identification of juvenile fluke residing in the intestine which are known to cause the pathology of infection responsible for paramphistomiasis as a clinical disease (Mason *et al.,* 2012; Millar *et al.,* 2012).

1.1.10 ***Treatment and control***

Oxyclozanide (a salicylanilide) is widely recognised as the most effective anthelmintic in the treatment of paramphistomosis demonstrating high efficacy in many studies with the ability to treat both immature and mature parasites (Malrait *et al.*, 2015). Despite its effectiveness against paramphistomosis, oxyclozanide remains unlicensed for use against rumen fluke in many countries (Rolfe & Boray, 1987; Sanabria *et al.*, 2014; Sanabria & Romero, 2008). Several treatment options such as febantel, niclosamide, resorantel, terenol and hexachlorophene have shown efficacy against paramphistomes, however these treatment options are also unlicensed for use in treatment of livestock (Rolfe & Boray, 1987; Rolfe & Boray, 1988) and so currently the main farm measure in rumen fluke control is the avoidance of grazing in intermediate host *G. trunculata* habitats. Grazing management has proven difficult due to variability depending upon farm layout, but once potential habitats have been successfully identified can be achieved through drainage, fencing and selective grazing (Forbes, 2017). However, changes in the climate also effect helminth control strategies, with high risk infection periods less predictable and the need for currently implemented strategies to be revised (Dijk *et al.*, 2010).

Many trematode species have developed resistance following inappropriate use of anthelmintics, thus, due to the increasing prevalence of *C. daubneyi* in the Western Europe new targets for drug compounds could be essential in the near future. Treatment and preventative methods for paramphistomosis is currently carried out through the use of a single anthelmintic – oxyclozanide, however this is not licensed for use against paramphistome infections in many countries (Arias *et al.*, 2013; Rolfe & Boray, 1988; Pinto *et al.*, 2019). The lack of a diagnostic test and suitable licensed

treatment options means research is greatly needed in order to treat paramphistomes in the future, with only a few anthelmintics available development of new treatment compounds is of significant importance (Pinto *et al.,* 2019). In order for suitable treatment options to be developed the gaps in knowledge of the parasites basic fundamental biology need to be explored and through the use of combined proteomic and transcriptomic techniques explored allowing development of diagnostic tests and subsequent treatment options (Huson *et al.,* 2017; Robinson *et al.,* 2009).

1.2 XENOBIOTIC DETOXIFICATION

1.2.1 *Introduction*

Helminths are capable of developing resistance to anthelmintics through a variety of mechanisms including the mutation of target genes, overexpression of efflux transport pumps and the overexpression of drug metabolising enzymatic systems (Alvarez *et al.,* 2005). Anthelmintic resistance has long been a worldwide issue in parasitic helminth species of veterinary importance (Wolstenholme *et al*., 2004). As stated above xenobiotic metabolizing enzymes (XMEs) from parasitic helminths have the potential to support the development of resistance (Cvilink *et al.,* 2008) and thus their identification in *C. daubneyi* could identify a route for drug resistance in the future and facilitate the need for new treatment strategies. Identification of anthelmintic resistance in a large number of livestock parasites has highlighted the need to develop methods of both chemical and non-chemical control which is now being addressed though investigation of the underlying mechanisms leading to resistance development (Kaplan, 2004; Laing *et al.*, 2013). It is clear that resistance to anthelmintics is a major threat to the global ruminant livestock industry (Waller, 1999). The continued inability to link mutations found in the anthelmintic protein target in the laboratory with field studies has renewed interest in other routes of resistance, such as anthelmintic metabolism (Vokral *et al.,* 2013), with some parasitic helminth studies correlating increased activity or levels of XMEs with resistance (Brennan *et al.,* 2007; Cvlink *et al.,* 2008). Prior to the accessibility of genomics in developing anthelmintics, targets were identified through molecular studies of genes hypothesised to be involved in parasite virulence (Brindley *et al.,* 2009). With readily available genomic studies, the fundamental biology of helminth parasites is now being resolved, this combined with

studies into RNAi in helminths it is thought it will allow new drug development and vaccines (Hotez *et al.,* 2008; Berriman *et al.,* 2007)

Understanding xenobiotic detoxification is crucial in understanding the methods through which metabolism of xenobiotics takes place. Most xenobiotics that enter an organism are metabolised enzymatically allowing their deconstruction to polar metabolites that can be easily excreted by the organism (Cvilink *et al.,* 2009). Metabolism of these xenobiotics takes place in three distinct phases, Phase I, Phase II and Phase III (Barrett, 1997) (**Table 1.2**). Members of Phase I and II are actively involved in detoxification, whilst members of phase III are involved in the active transport of substances. The majority of research into xenobiotic detoxification has been carried out in mammals, whilst helminths are still largely unstudied. Although increased number of members of the Phase I flavin containing monooxygenases (FMOs) and Phase II drug metabolism soluble glutathione transferases (GSTs) have been detected in resistant isolates of *Fasciola hepatica* (Scarcella *et al.,* 2012). Development of an understanding of helminth detoxification could assist in the development of new treatment compounds (Brophy *et al.,* 2012).

Table 1.2: Phases I and II detoxification enzymes utilised in the biotransformation of xenobiotics (Parkinson, 1996).

REACTION	ENZYME	LOCALIZATION
	Phase I	
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Reductive dehalogenation	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes
	Phase II	
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Cytosol, microsomes
	Amino acid conjugation	Mitochondria, microsomes
	Acylation	Mitochondria, cytosol
	Methylation	Cytosol, microsomes, blood

1.2.2 ***Phase I detoxification***

Oxidation, hydrolysis, or reduction of drugs are the main components of Phase I detoxification. Cytochrome P450 (CYP) has been described as the essential enzyme in phase I of detoxification, with thousands of isoforms identified in research across different organisms with each classified into families and sub families based on their sequence homology. Flavin containing monooxygenases (FMOs) are also important in phase I detoxification with a main role in oxidation of substrates with nucleophilic nitrogen or sulphur atoms, whilst this family is a lot smaller and less researched than the CYPs it is still important in the biotransformation of xenobiotics (Cvilink *et al.,* 2009; Krueger & Williams, 2005). Monoamine oxidases (MAO) represent another important phase I detoxification enzyme capable deamination of amine containing xenobiotics (Lang & Kalgutar, 2003). FMOs, CYPs and MAOs represent the three phase I detoxification enzymes analysed in this study.

1.2.3 ***Cytochromes P450 (CYP)***

Cytochrome P450s (CYP) are a superfamily of enzymes that have been described in almost all living organisms (Nelson *et al.,* 1993), representing a widespread family of mono-oxygenases involved in drug biotransformation (Ortiz de Montellano, 2005). Studies of CYPs in helminths have been neglected due to the intensive study of the Phase II GSTs, however the role of CYPs is now becoming clearer with several studies identifying helminths with active CYPs (Brophy *et al.,* 2012; Alvinerie *et al.,* 2001; Matouskova *et al.,* 2016), with helminth genomics predicting hundreds of potential Phase I hydrolases and reductases to be present (Dieterich *et al.,* 2008). The model nematode *C. elegans* has had its CYPs extensively studied, unveiling an upregulation with treatment of xenobiotics (Kulas *et al.,* 2008). Due to its role as a main Phase I component in vertebrates also identified to have roles in house-keeping cellular

metabolism (Brophy *et al*., 2012). CYPs are known to have peroxidase or reductase activity as well as in metabolism of endogenous compounds (Skalova *et al.,* 2010). The families of CYP have been found to have a range of roles with CYP1, 2 and 3 involvement in drug metabolism and further families having house-keeping roles (Omari & Murry, 2007). Nelson *et al*, 2009, compared the number of CYP450 genes in a range of organisms including studied helminths and are detailed in **Table 1.3.**

Table 1.3: Number of CYP genes in studied helminth species, adapted from initial publication by Nelson *et al.,* 2009.

Organism	***Number of CYPs***
Schistosoma mansoni	1
Schistosoma japonicum	1
Schistosoma haematobium	1
Opisthorchis felinus	1

It has been suggested that parasitic flatworms may only have a single CYP gene possibly representing a simplification of detoxification and adaptation to survival (Berriman *et al.,* 2009; Pakharukova *et al.,* 2012). CYPs have been noted as a possible target for development of new therapeutic agents against trematodiases due to their widespread presence (Mordvinov *et al.,* 2017).

1.2.4 *Flavin-containing mono-oxygenase (FMO)*

FMOs represent an ancient conserved family that has been identified in almost all phyla researched (Petalcorin *et al.,* 2005). As a family FMOs are considerably smaller than the CYPs, with their mechanism of action still poorly understood (Cvilink *et al.,* 2009). Regardless of the information available about the super family, FMOs have previously been identified as the key detoxification mechanism in *F. hepatica,*

facilitating the development of resistance to triclabendazole (Alvarez *et al.*, 2005), and are known to be involved in the oxidation of a range of xenobiotic compounds acting as important mediators of biotransformation (Krueger & Williams, 2005). Studies show FMOs to be different from other monooxygenases as its active oxygenating species is able to oxidize any nucleophile in contact with the active site (Petalcorin *et al.*, 2005).

1.2.5 *Monoamine oxidase (MAO)*

In contrast to CYPs which have over 200 primary sequences identified, there are two known MAO isozymes, MAO-A and MAO-B, differing in their substrates and sensitivity to inhibitory compounds (Shih *et al.*, 1998; Benedetti, 2001). Research into MAOs has been largely overlooked due to the research carried out on CYPs, however its importance and contribution to detoxification is well known (Benedetti, 2001). Monoamine oxidases have previously been reported in several of the platyhelminthes such as *Hymenolepis diminta* (Ribeiro & Webb, 1984).

1.2.6 *Phase II detoxification*

Phase II of detoxification mainly involves enzyme families capable of catalysing conjugation reactions often sequential to the initial oxidation, reduction or hydrolysis by the phase I enzymes (Iyanagi, 2007). The three most studied of the phase II enzymes are the glutathione transferases (GSTs), UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) and so will be the specific protein families considered in this study.

1.2.7 *Glutathione Transferase (GST)*

As a superfamily the GST enzymes are a ubiquitous family of proteins consisting of transferases, peroxidases isomerases and thioltransferases, with a main function in

detoxification (Umasuthan *et al.*, 2012; Yang *et al.*, 2019). GST are primarily involved in phase II detoxification through their mechanism of action catalysing the conjugation of glutathione to the electrophilic centre of a substrate leading the production of soluble detoxified peptide derivatives that can be the secreted by the enzymes involved in phase III of detoxification (Frova, 2006; Scarcella *et al.*, 2012). GSTs have been described in many parasites and have been identified as essential for helminth survival through there mechanisms of detoxification against xenobiotic compounds and involvement in immunomodulation (Torres-Rivera & Landa, 2008), with previous investigations describing the absence of CYP detoxification mechanisms (Precious & Barrett, 1989), GSTs have been extensively studied in many species. Mammalian GSTs can be further divided into three families, (1) cytosolic, (2) microsomal, (3) plasmid-encoded bacterial fosfomycin resistant GSTs (Frova, 2006). Classes of GST are assembled based on N-terminal amino acid sequences and are divided into 13 groups, alpha, beta, kappa, mu, theta, zeta, epsilon, sigma, omega, pi, tau, phi, delta. Research has identified the importance of GSTs in the development and establishment of chronic infection of helminths with their role in detoxification and ligand binding (Barrett, 2001).

1.2.8 ***UDP-glucuronosyltransferase (UGT)***

UDP-glucuronosyltransferases (UGTs) are actively involved in phase II of detoxification conjugating xenobiotics and leading to their degradation to metabolites, through their ability to add UDP-glucuronic acid to xenobiotics. This addition enhances solubility and in turn eliminates the xenobiotic (Buckley & Klaassen, 2009). UGTs represent a vital detoxification super family in the phase II biotransformation enzymes (Buckley & Klaassen, 2009). Phase II detoxification enzymes in particular have been identified in playing a role in development of resistance in helminths, with

particular focus on the role of UGTs in this development (Vokral *et al.,* 2013). Many anthelmintics such as benzimidazole have been shown to be detoxified through glycosylation, with glyosidation increased in resistant helminth strains, leading to further interest in UGTs role in development of resistance (Laing *et al.,* 2010). Recent developments in genome sequencing have elucidated the UGT multigene superfamily in gastrointestinal parasites of ruminant livestock (Matouskova *et al.,* 2018), allowing their comprehensive study using bioinformatic techniques.

1.2.9 ***Sulfotransferase (SULT)***

Another mechanism through which xenobiotics can become detoxified is through sulfotransferases ability to carry out sulfuryl transfer in which a sulfo group is donated from 5'-phosphoadenosine-3'-phosphosulfate (PAPS) to the hydroxyl group of a substrate (Gamage *et al.,* 2005) and was first described by Baumann, 1876. Conjugation of sulphate to these molecules can lead to inactivation of the compound or result in increasing solubility allowing its excretion (Falany, 1997). In humans, two main classes of SULTs have been identified and compared with UGT's, little is known about the mechanisms through which SULTs metabolise their substrates, which may be due to SULT being a secondary metabolic pathway of phase II detoxification (Gamage *et al.,* 2005).

1.2.10 ***Phase III detoxification***

The final stage in xenobiotic detoxification is via transporters with ability to transport toxic compounds across membranes, generally known as phase III of detoxification (Cvilink *et al.,* 2009). There are two main types of transporter involved in phase III detoxification - transporters involving import of xenobiotics and transporters

involving export of them and metabolites produced during detoxification (Cvilink *et al.,* 2009), with ABC transporters known to play an important role through hydrolysation of ATP releasing energy allowing the active transportation of compounds derived from phase I and II detoxification (Laing *et al.*, 2013). Notably, ABC transporters are regulated through exposure with specific inducers in line with observed expression of phase I and phase II enzymes emphasising their importance in protection from xenobiotic compounds (Xu *et al.,* 2005). It is important to also note that further metabolism may take place prior to excretion (Barrett, 2011).

1.3 EXTRACELLULAR VESICLES

1.3.1 Introduction

Extracellular vesicles (EVs) are a heterogenous population of membrane bound vesicles enclosed within lipid bilayers released by almost all cell types (Willms *et al.*, 2018). First identified in platelet-free sera (Chargaff & West, 1946), early studies identified them as a mechanism of removal for unwanted cellular materials and by-products of metabolic pathways, however research has now been identified as key components in intercellular communications (Thébaud & Stewart, 2012). Despite their initial characterisation, EVs have now been found capable of mediating signals and immune responses through their ability to store bioactive molecules such as proteins, lipids and nucleic acids as cargo (Marcilla *et al.*, 2014; Twu & Johnson, 2014; Raposo *et al.*, 1996; Valadi *et al.*, 2007; Subra *et al.*, 2010; Torre-Escudero & Robinson, 2017). The mechanisms of communication via EVs is still not fully understood, however several potential mechanisms of action have been suggested. It is hypothesised that membrane bound surface receptors allow targeted delivery to recipient cells, with identification of a range of transmembrane proteins with soluble components contained internally, allowing their content to make changes to the recipient cells physiological state (Yanez-Mo *et al.*, 2015; Thery *et al.*, 2009).

Research into EVs in parasitic organisms has recently gained significant attention, with studies showing parasite derived EV activity on the host immune response (Buck *et al.*, 2014), including pathogenicity directed towards host cells (Chaiyadet *et al.*, 2015). EVs have been identified as novel mediators of communication, once released they can be absorbed by recipient cells where they deliver their cargo molecules and are able to regulate a range of biological processes, through release of internalised

biological components (Valadi *et al.*, 2007; Veerman *et al.*, 2019). Parasites evolution within their hosts has led to the development of cross-species communication, with recent reports detailing the possible role of EVs in facilitating communication, not only parasite-parasite but also parasite-host (Coakley *et al.*, 2017) with numerous parasite studies detail their release by trematodes (**Table 1.4**). EVs have been identified in the ES products of many helminth species, believed to transfer parasite factors to the hosts cells (Buck *et al.*, 2014), with experimental evidence of their ability to modulate gene expression due to their containment of small non-coding RNAs and proteins.

Table 1.4: Identification and study of EVs release by helminth species (- indicates information not available) (Kifle *et al.*, 2017).

Helminth	Type of vesicle	EV origin	Cargo composition characterized	EVs target	Applied
F. hepatica	Exosome-like vesicle	Adult worms	Proteins	Uptake by intestinal cells	*In vitro*
F. hepatica	Exosome-like vesicle	Adult worms	Proteins, miRNAs	-	-
F. hepatica	EVs	Adult worms	miRNAs	-	-
D. dendriticum	Exosomes	Adult worms	Proteins and miRNAs	-	-
S. japonicum	Exosome-like vesicles	Adult worms	Proteins	Macrophage	*In vitro*
S. japonicum	Exosome-like vesicles	Adult worms	Proteins, miRNA	Uptake by mouse liver cell	*In vitro*
S. mansoni	Exosome-like vesicles	Adult worms	Proteins	-	-
S. mansoni	EVs	Schistosomules	Proteins, miRNAs	-	-
O. viverrini	EVs	Adult worms	Proteins	Uptake by human cholangiocytes	*In vitro*
E. caproni	Exosomes	Adult worms	Proteins	Uptake by intestinal cells	*In vitro*
E. caproni	Exosomes	Adult worm	-	Systemic blood	*In vivo*
E. multilocularis	Vesicles derived from metacestodes	Metacestodes	-	Mononuclear cells/ dendritic cells	*In vitro*
E. granulosus	Exosomes	Hydatid cyst	Proteins	-	-
T. crassiceps					
M. corti	EVs	Larvae	Protein and miRNAs	-	-
E. multilocularis					

Table 1.4: *continued.*

H. polygyrus	Exosomes	Intestinal tract of adult nematode	Proteins, mRNAs, small RNAs and Y RNAs	Intestinal epithelial cells of the host	*In vivo and in vitro*
H. polygyrus	EVs	Adult/larval worms	-	Uptake by macrophage	*In vivo and in vitro*
B. malayi	Exosome-like vesicles	Larval stage	Protein and miRNA	Internalization by macrophage	*In vitro*
T. suis	EVs	Larvae	miRNA	-	-
T. circumcincta	Exosome-like vesicles	Larvae	Proteins	Immunoglobulins	*In vitro*
T. muris	Exosome-like vesicles	Adult worms	Proteins, mRNAs and miRNAs	Uptake by murine colonic organoids	*In vitro*

1.3.1 ***Characterisation, morphology and isolation***

Although the study of EVs has grown substantially, studies remain hampered due to the multitude of methods utilised for isolation and characterisation. Therefore, a standard, efficient and reproducible method of isolation is key to allowing studies to be comparable in the future (Furi *et al.,* 2017). Environmental conditions, and cellular source are hypothesised to be responsible for the size, content and membrane composition of EVs released, with the three currently defined subpopulations – apoptotic bodies, microvesicles and exosomes, predominantly characterised through their mechanisms of biogenesis. Microvesicles are described as shed from the plasma membrane (100-800 nm), apoptotic bodies are shed through cells undergoing programmed cell death (200 nm-5 μm) and exosomes which are released from the endosomal pathway (10-150 nm) (Lasser *et al.,* 2018) (**Table 1.5**).

EVs are comprised of mixed populations, however studies are now beginning to show differences in EV composition through modifications of culture conditions *in vitro*, allowing stimulation of different host environments/physiological states (Kuchaezewska, 2013). EV morphology is also subject to alteration through isolation method with analysis of unprocessed samples identifying 11 variations of membrane vesicles with only 59% observed to be true to the originally thought spherical single membrane bound type (Hoog & Lotvall, 2015). Identification of isolated populations is currently carried out mainly through the use of transmission electron microscopy (TEM). However, characterisation can also be carried out through western blotting and size particle tracking (Gardiner *et al.,* 2016).

Table 1.5: Characterisation of exosomes, microvesicles and apoptotic bodies. Adapted from Mathivanan *et al.,* (2010).

	Exosomes	**Microvesicles**	**Apoptotic bodies**
Diameter (nm)	30-100 nm	50-1,000 nm	100-5,000 nm
Flotation density (rate zonal centrifugation)	1.10-1.21 g/mL	-	1.16-1.28 g/mL
Morphology	Cup-shaped	Various shapes	Heterogeneous
Lipid composition	low phosphatidylserine exposure, cholesterol, ceramide, contains lipid rafts, sphingomyelin	High phosphatidylserine exposure, cholesterol	High phosphatidylserine exposure
Protein markers	ALG-2 interacting protein 1 protein, Tumour susceptibility gene 101, Heat shock cognate 70, CD63, CD81, CD9	Selectins, integrins, CD40, metalloproteinases	Histones
Site of origin	MVBs	Plasma membrane	-
Mode of extracellular release	Constitutive and regulated	Regulated	Regulated
Mechanism of discharge	Exocytosis of MVBs	Budding from plasma membrane	Release from blebs of cells undergoing apoptosis
Composition	Proteins, DNA, miRNA, mRNA	Proteins, miRNA, mRNA	Proteins, DNA, miRNA, mRNA

Comprehensive research has been carried out on the protein composition of EVs detailing the contents of individual sub-populations from an assortment of cell lines (Beckler *et al.,* 2016; Gonzalez-Begne *et al.,* 2009). However, due to the multitude of isolation techniques utilised, culture conditions and cell line origins it is difficult to standardise the protein composition of each sub-type. Instead, there are a variety of proteins found to be common dependent upon the mechanism of release (Abels & Breakfield, 2016). ExoCarta is a database of EV studies containing proteomic and transcriptomic data resolved on different populations of EVs from a variety of organisms, each protein resolved is ranked allowing identification of proteins found to be consistent across all studies allowing a means of characterising EVs through proteins present representing reliable protein markers (**Figure 1.4)**.

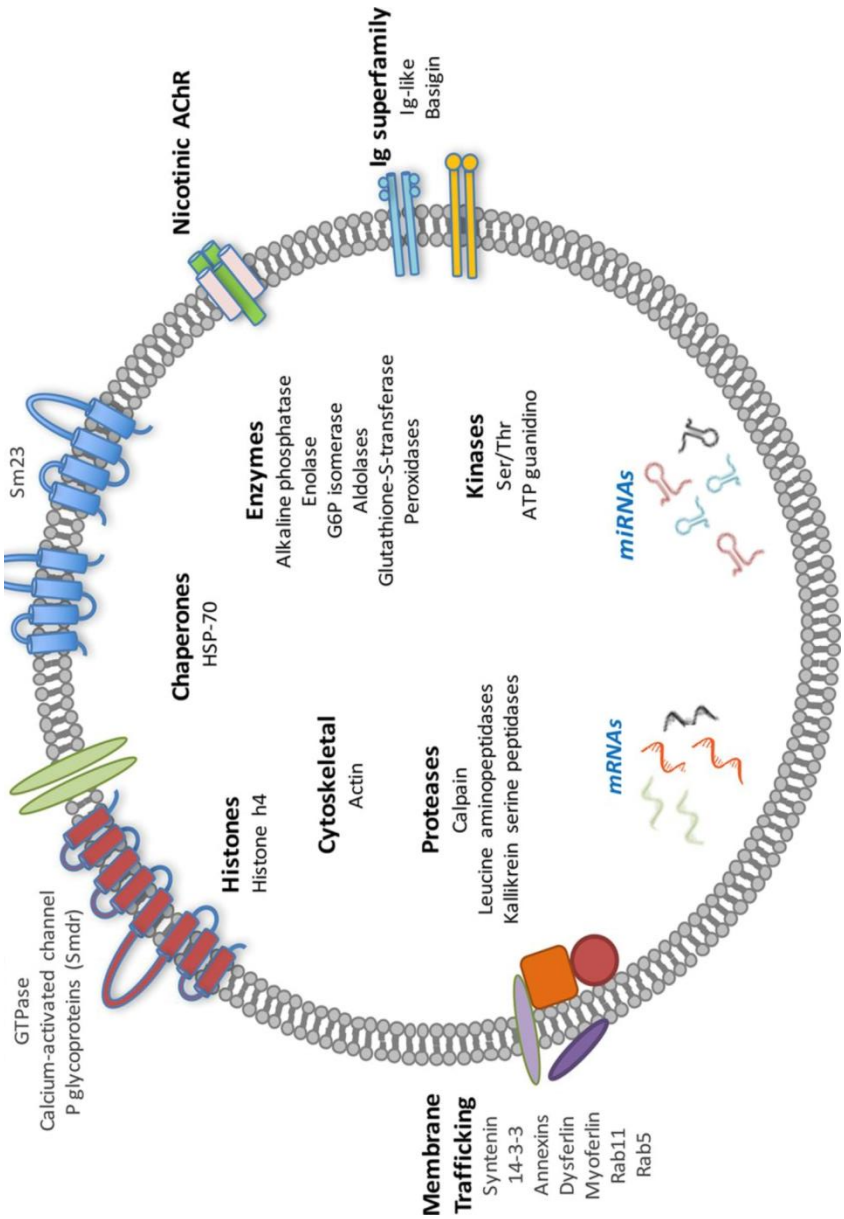


Figure 1.4: Schematic representation of EV proteins of interest in parasitic helminths (Kifle *et al.*, 2017).

Differential centrifugation (DC) is currently the most utilised method of EV isolation, however, there are many mechanisms that can be utilised in their isolation that can be divided into five distinct categories – differential centrifugation, density gradient separation, polymer-based precipitation, immunoselection and microfluidic isolation (Greening *et al.,* 2017; Gardiner *et al.,* 2016). All isolation methods can be utilised individually or in combination allowing EVs to be purified from an array of biological samples (Furi *et al.,* 2017). Differences in both size and sedimentation properties allows isolation of subpopulations by differential centrifugation (Cvjetkovic *et al.,* 2014), which remains the gold standard in EV isolation. Spin force utilised in allowing isolation of subpopulations is shown in **Table 1.6**. However, despite the size-related subpopulations of EVs the definitive boundaries of subgroups and purity of samples still remain unclear (Guerreiro *et al.,* 2018).

Table 1.6: Differential centrifugation (DC) spin speed required for purification of EV subpopulations (Crescitelli *et al.,* 2013).

Subpopulation	**Spin force × *g***
Apoptotic bodies	2000-10,000 × *g*
Microvesicles	10,000-20,000 × *g*
Exosomes	>100,000 × *g*

1.3.2 ***EV Biogenesis***

Formation of specific EV sub-populations takes place through various mechanisms, exosomes (**Section 1.3.3**) through the formation and subsequent release of mutli-vesicular bodies and microvesicles (**Section 1.3.4**) through direct budding of the membrane (**Figure 1.5**) (Raposo & Stoorvogel, 2013).

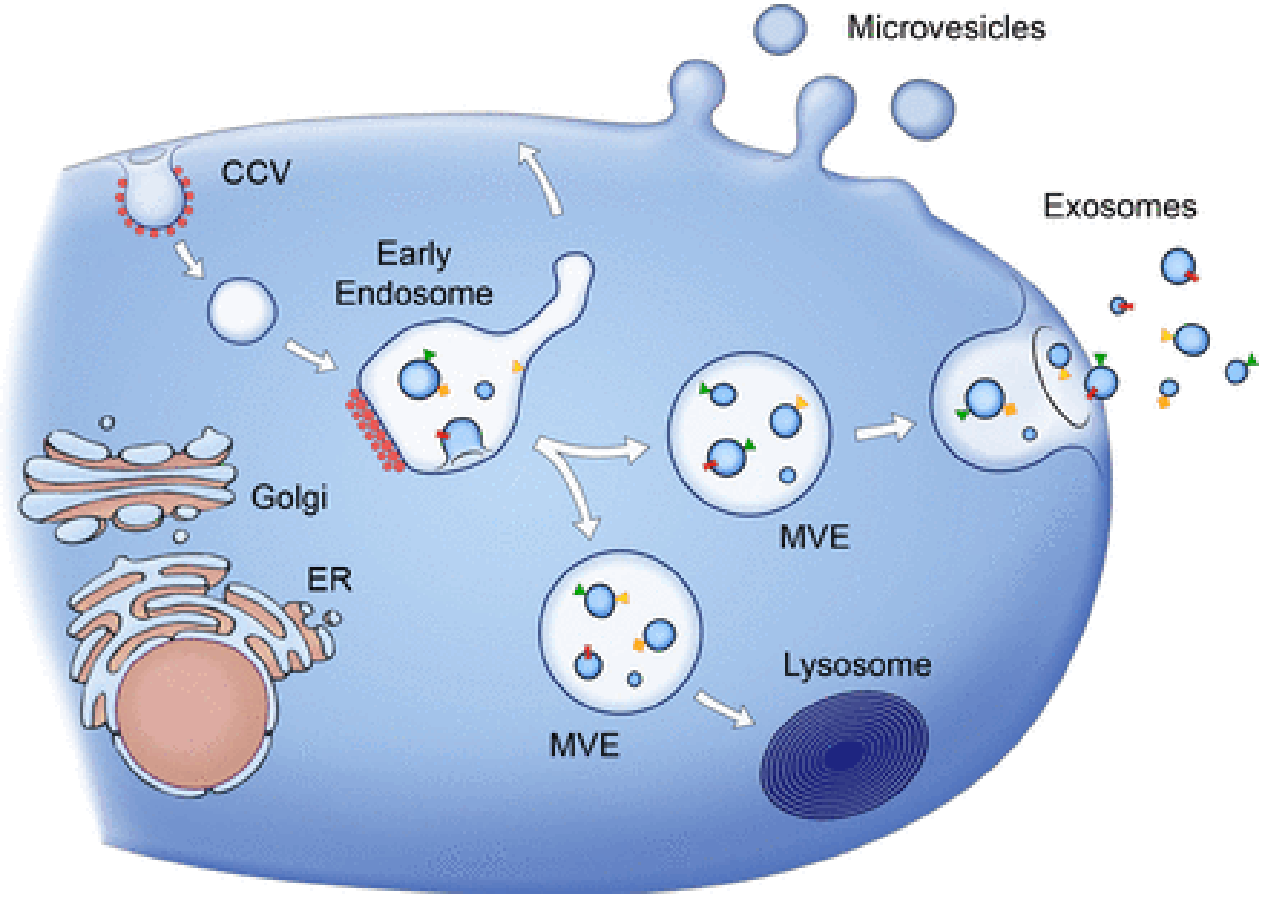


Figure 1.5: Extracellular Vesicle (EV) formation. Microvesicles bud directly from the plasma membrane whilst exosomes are formed by budding to early endosome and subsequent release by fusion of the formed MVE to the plasma membrane through with ESCRT-dependent or ESCRT-independent mechanisms (Raposo & Stoorvogel, 2013).

1.3.3 ***Exosomes***

Exosome formation starts with the inward budding of the late endosomal membrane creating multivesicular bodies (MVBs) that contain numerous intraluminal vesicle (ILVs), these vesicles are subsequently released as exosomes following fusion of the MVB with the plasma membrane (Torro-Escudero *et al.,* 2016). This process is driven by the endosomal sorting complex required for transport (ESCRT) machinery (Torro-Escudero *et al.,* 2016). This machinery can be divided into 4 groups, ESCRT-0, -I, -II and -III. ESCRT -0, -I and -II are responsible for the migration of ubiquinated proteins towards the endosome whilst ESCRT -III is involved in invagination of the membrane forming the ILV (Schorey *et al.,* 2014). Finally, AAA-ATPase VPS4 is required for ESCRT disassembly allowing completion of invagination (Hasegawa *et al.,* 2011). However, there have also been a number of ESCRT-independent pathways described in the mechanism of exosome biogenesis. It is proposed there are 4 possible mechanisms leading to exosome formation as described by Colombo *et al.* (i) The ESCRT pathway, (ii) the lipid pathway, (iii) the tetraspanin pathway and (iv) a hybrid method comprised of mechanisms from (i), (ii) and (iii), with the final fusion of the MVBs required for release of ILVs regulated through a selection of small GTPases (Chen *et al.,* 2001; Savina *et al.,* 2004).

1.3.4 ***Multivesicular bodies***

In contrast, MVs are the result of direct budding of the plasma membrane initiated through external stimuli changing Ca^{2+} levels and membrane remodelling (Hugel *et al*., 2005; Muralidharan-Chari *et al*., 2009). Scramblase is activated by the changes in Ca^{2+} leading to phosphatidylserine translocation to the outer membrane, causing asymmetry thought to cause curvature of the membrane and rearrangement of the cytoskeleton by calpain (Fox *et al.,* 1990). Studies also suggest the involvement of

GTPases such as ADP-ribosylation factor-6 (ARF6), activating proteases in response to extracellular signal-related kinase (ERK) (Muralidharan-Chan *et al*., 2009). Abscission of the membrane is not fully understood; however, hypothesis suggest involvement of VPS4 as in the ESCRT pathway (Booth *et al*., 2006).

1.3.5 ***Interaction with recipient cells***

EVs have been shown to travel significant distances through diffusion allowing entry to biological fluids (Kucharzewska & Belting, 2012). Several methods of EV-associated cargo release have been described including membrane rupture leading to release of internalised cargo (Taraboletti *et al.*, 2006), interaction with plasma membrane receptors and fusion with the plasma membrane/endocytosis allowing direct interaction with signalling machinery (Svensson *et al.,* 2011). Exosomes and MVs are both used as means of communication for host-parasite interaction and both represent energy driven processes. However, exosomes appear to be constitutively released whilst MV release is initiated by external stimuli (Angelot *et al.,* 2009). Three main mechanisms of EV interaction with recipient cells leading to activation of signalling have been described, the first described through the interaction with membrane bound proteins interacting with target cells in a juxtracrine fashion leading to activation of the target cell, the second involving the cleavage of membrane proteins by proteases producing ligands capable of interacting with target cell receptors and the final describing fusion of the EV with the target cell leading to the transfer of all EV proteins and RNA into the recipient cell (Mathivanan *et al.,* 2010) (**Figure 1.6**).

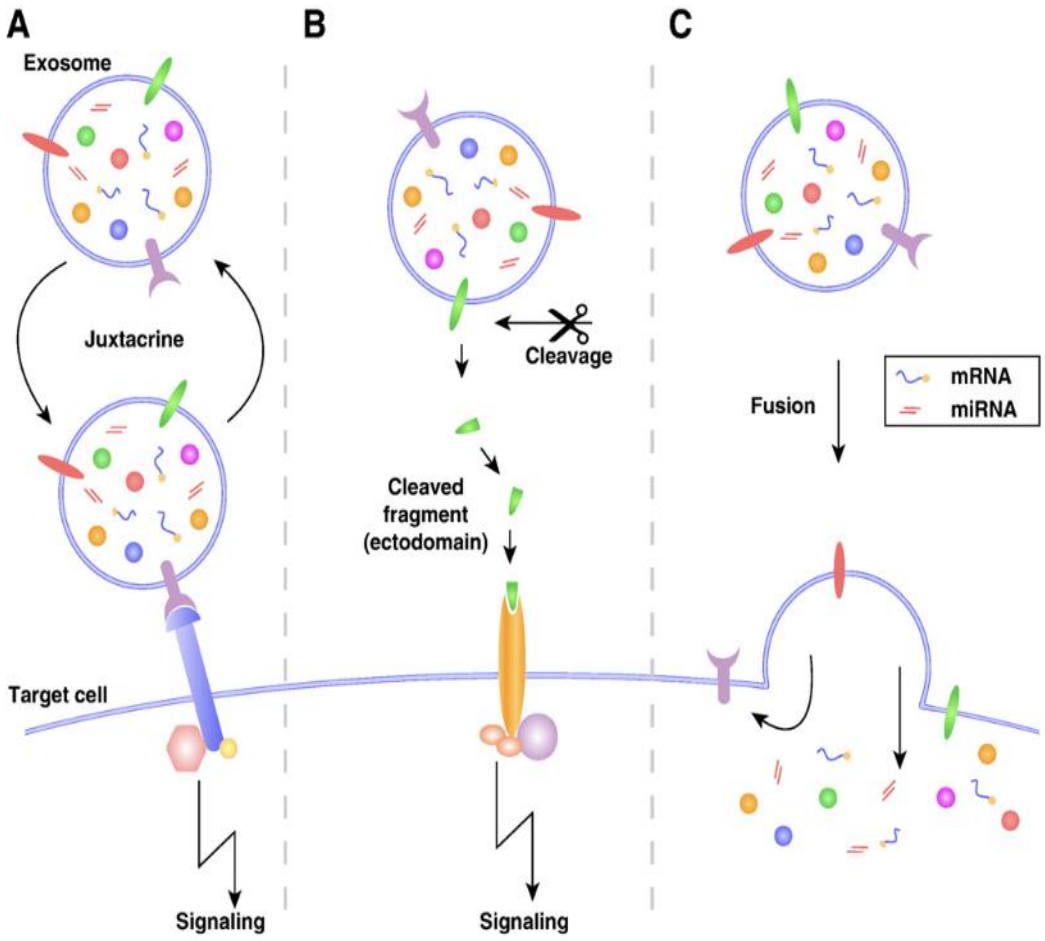


Figure 1.6: Potential mechanisms of EV interaction with recipient cells. A – Membrane proteins interact with target cell receptors activating signalling in a juxtracrine fashion. B – Membrane proteins are cleaved by proteases and act as soluble ligands binding to surface receptors leading to activation of signalling, C – EVs fuse with target cells releasing their internalised cargo into the recipient cell allowing activation of signalling in the recipient cell through release of mRNA, miRNA and proteins (Mathivanan *et al.*, 2010).

1.3.6 ***EVs as anti-parasite therapy***

Development of anthelmintic targets prior to improvements in omics technologies was carried out through molecular investigations of genes involved in parasite virulence (Brindley *et al.,* 2009). Developments in these technologies has led to the resolution of numerous parasitic helminths genomes, assisting developments in understanding of the parasite's biology and allowing the elucidation of novel drug targets and potential vaccine candidates (Brindley *et al.,* 2009; Cuesta-Astroz *et al.,* 2017). The overuse of antiparasitic treatments has added to growing concerns of drug resistance and a need for further investigation into novel treatment options (Clay et al., 2014), with EVs recently identified as potential targets for therapeutics (Lasser et al., 2018). Parasite devised EVs have demonstrated modulatory effects on host immune systems as well as pathological effect on host cells (Buck *et al.,* 2014; Wang *et al.,* 2015). Depending on the cellular source of EV release, they have been found capable of immune stimulation/activation which could be exploited in the future development of treatments and vaccinations through disruption of host communication (Eichenberger *et al.,* 2018). Proteins involved in EV biogenesis pathways are now well documented and allow elucidation of key regulators of their formation in parasites through transcript and genome analysis. EV biogenesis proteins could be a key in the future of parasite control as inhibition of their release could prevent the parasites' successful establishment (Torre-Escudero *et al.,* 2016). Physiological and pathological conditions are influenced by EVs through their mechanisms of bioactive particle delivery making them important mediators of communication and an interesting area of research as potential therapeutic targets as well as in the delivery of therapeutic agents (Wiklander *et al.,* 2019).

1.4 THESIS AIMS AND OBJECTIVES

Given the current absence of information available on its fundamental biology and the rapid development of resistance to treatment options in many helminth species, the focus of this thesis will be the study of the neglected trematode parasite *Calicophoron daubneyi* which is quickly emerging as a common parasite of ruminants in Western Europe.

The specific aims of this project are to:

- Resolve the soluble somatic and egg proteome of *C. daubneyi* allowing identification of enriched protein families and potential diagnostic markers and vaccine candidates.
- Isolate EVs from *C. daubneyi* ES products and perform proteomic profiling in order to identify proteins potentially involved in modulation of the immune response, and successful establishment within the host.
- Investigate the specific EV surface proteins and their potential interaction in successful parasite establishment within the host.
- Mine the *C. daubneyi* transcriptome to elucidate protein families involved in anthelmintic detoxification, utilising characterised detoxification proteins from closely related helminth species.
- Investigate the specific detoxification protein families expressed in the soluble somatic, egg and EV proteomes of *C. daubneyi* allowing elucidation of potential drug targets.

CHAPTER 2.

GENERAL MATERIALS AND METHODS

2.1 Parasite collection and in vitro maintenance

Rumen Fluke parasites were collected from cattle found to be naturally infected at a local Welsh abattoir (Randall Parker Foods, Llanidloes, UK). Rumen fluke samples were collected between 2015 and 2017, with 3 samples from each infected rumen randomly selected to undergo species identification. These samples were acknowledged as representative of all parasites collected. Prior to culture each batch of 40 parasites were placed in phosphate buffered saline (PBS) at a consistent temperature of 39^{o}C facilitating removal of host contamination. Parasites were stored in 1 ml of PBS per fluke upon return to the laboratory and the PBS retained allowing collection of eggs. A further 15-minute wash in PBS was carried out prior to addition of 1 ml per fluke of DME culture media (DMEM) supplemented with 15 mM HEPES, 61 mM glucose, 2.2 mM calcium acetate, 2.7 mM magnesium sulphate, 1 µM serotonin and gentamycin (5 µg/ml) and incubated for 6 hours at 39^{o}C. Following culture, parasites were removed from media and 'snap-frozen' in liquid nitrogen with both parasites and culture media subsequently stored at -80^{o}C for further analysis.

2.2 Identification of *C. daubneyi* eggs

All eggs used in experimentation were collected fresh from the PBS washes carried out on parasites prior to culture. PBS washes were retained and washed through a series of 300 µm, 150 µm, and 45 µm mesh sieves, allowing removal of debris and isolation of eggs. Eggs were collected on the 45 µm sieve and washed with ddH_2O into a measuring cylinder and left for 10 minutes to sediment. The resulting supernatant was aspirated and repeated 3 times allowing further removal of debris. Resulting samples were submitted to light microscopy to confirm them as *C. daubneyi* eggs and undeveloped before storing for further processing (Kajugu et al., 2015)

(**Figure 2.1**). Eggs deemed to be at the correct stage were kept in the dark at 4^{o}C in order to ensure they were not embryonated before processing.

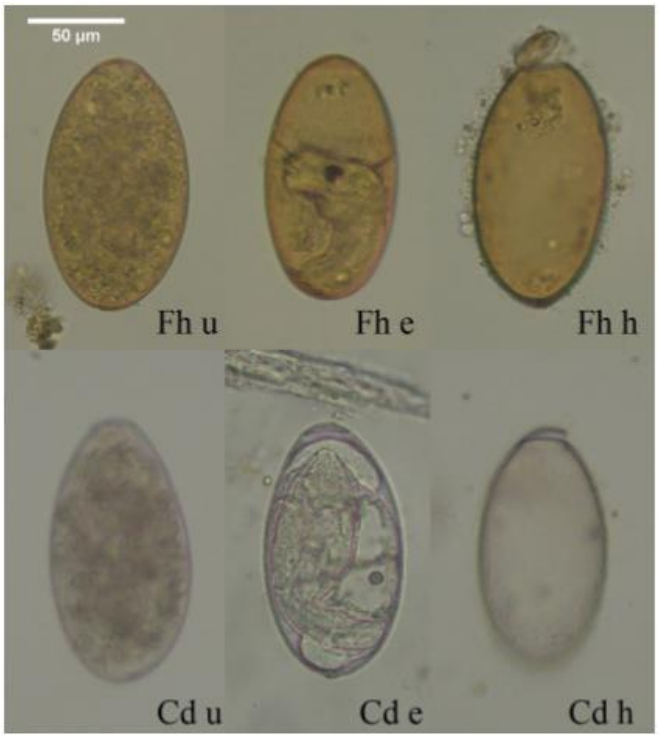


Figure 2.1: Comparative analysis of *C. daubneyi* (Cd) and *Fasciola hepatica* (Fh) eggs. (u) – undeveloped, (e) – embryonated and (h) – hatched (Chryssafidis *et al.*, 2015).

2.3 Adult *C. daubneyi* soluble somatic preparation

Samples stored at -80^{o}C were defrosted on ice and homogenised in batches of 10 in a homogenisation buffer containing 20 mM potassium phosphate (pH 7.4), 0.1% v/v Triton-X 100 and a mini cOmplete protease inhibitor tablet (Roche, U.K.). Homogenisation was achieved utilising a glass homogeniser contained within an icebox. Soluble samples were obtained though homogenate centrifugation at 100,000 × *g* for 45 minutes at 4^{o}C in a mini ultra-centrifuge (S55-S rotor, Sorval MX120 centrifuge, Thermo scientific). The resulting supernatant was retained, and the fat layer and pellet discarded. Soluble samples were then precipitated out using TCA and acetone (**Section 2.6**) before being re-solubilised in a desired buffer as detailed by Morphew et al. (2014).

2.4 Egg preparation

Isolated eggs were submitted to initial centrifugation at 2,000 × *g* at 4°C allowing eggs to be pelleted and the supernatant removed. Eggs were resolubilised following the method of Moxon *et al.* (2010), in homogenisation buffer (20 mM potassium phosphate pH 7.4, 0.2% v/v Triton-X 100 and cOmplete mini EDTA-free protease inhibitor (Roche, U.K). Eggs were placed in a mortar and pestle cooled through the addition of liquid nitrogen and homogenised. Debris was removed from the sample through centrifugation at 14,000 × *g* at 4°C for 1 minute and the supernatant containing the soluble protein extract retained and stored at -20°C for future analysis.

2.5 Extracellular Vesicle collection – Differential Centrifugation (DC)

Following *C. daubneyi* culture media was retained and EVs purified from the ESP contained following the method of Marcilla *et al.* (2012). Culture media was initially centrifuged at 300 × *g* for 10 minutes at 4°C and then at 700 × *g* for 30 minutes at 4°C allowing removal of large host contaminants and debris. The supernatant was subsequently submitted to ultracentrifugation at 100,000 × *g* for 80 minutes at 4°C (Optima L-100 XP ultracentrifuge (Beckman Coulter, U.K.)) and the ES supernatant removed. The remaining pellet was washed in 5 ml of PBS and agitated until suspended. The sample was again submitted to ultracentrifugation for 80 minutes at 4°C and the supernatant discarded. The resulting pellet was re-suspended in 200 µl of PBS and stored at -80°C for future experimentation.

2.6 TCA Precipitation

Trichloroacetic acid (TCA) protein precipitation was carried out following the protocol described by Link & Labaer (2011). Sample was added to ice-cold 20% TCA w/v in acetone at a 1:1 ratio, mixed and stored at -20°C for one hour. Following incubation, samples were centrifuged at 21,000 × *g* at 4°C for 15 minutes allowing precipitated protein to be pelted. The resulting supernatant was discarded, and the

pellet washed twice in 200 µl of ice-cold acetone followed by centrifugation at 21,000 × *g* for 15 minutes. The final protein pellets were left to dry at -20^{o}C and resuspended in Buffer Z (8 M urea, 2% w/v CHAPS, 33 mM DTT, 0.5% v/v ampholytes).

2.7 Bradford protein estimation

Purified cytosolic and egg fractions were quantified using the method of Bradford (1976). Bradford reagent (Sigma, U.K) was used following the manufacturer's instructions. In brief, 1 ml of Bradford reagent, 28.3 µl of ddH_2O and 5 µl of sample were added to a cuvette, mixed and analysed on a Cary 50 Bio UV-visible spectrophotometer (Varian, U.K.) at a wavelength of 595 nm. The 5 µl of sample was substituted with an equal volume of buffer as a control blank in order to counteract the possible interaction of buffer with Bradford reagent. Concentrations of Bovine Serum Albumin (BSA) were utilised in the calibration of the assay creating a standard curve with a range of 0.2-1.6 mg/ml

2.8 Preparation of SDS page gels

A Mini Protean III kit (Bio-rad, U.K) was used in the casting of 7 cm × 7 cm, 12.5% polyacrylamide gels, with glass plates assembled following the manufacturer's instructions. Resolving gel was prepared by combining the components listed in (**Table 2.1)** and pipetted into the gel cast with a layer of water-saturated isobutanol added before allowing to set for 30 minutes. Once set the isobutanol was removed, the gel was washed with ddH_2O and dried with filter paper. Stacking solution was then added to the top of the stacking gel and left to set before being rinsed again with ddH_2O (**Table 2.1**). For 1D gels, a comb was added and left to set for a further 30 minutes. For 2D gels, a layer of water-saturated isobutanol was again added before being left to set.

Table 2.1: Components and volumes to create stacking and resolving gel for casting 12.5% 1D and 2D polyacrylamide gels (each creates two gels).

Components	***Stacking gel***	***Resolving gel***
Acrylamide	4210 µl	375 µl
Gel buffer	2500 µl	625 µl
ddH_2O	3340 µl	1500 µl
Ammonium persulphate	37.5 µl	12.5 µl
TEMED	10 µl	3.75 µl

2.9 1-Dimensional SDS-PAGE

Once set the well-comb was removed from the stacking layer and the gels assembled into the Mini-Protean III tank with the inner and outer chambers were filled with Tris-glycine SDS (TGS) buffer (Bio-Rad, U.K.). Sample concentrations were quantified using the method of Bradford (Bradford, 1976). Appropriate volumes of sample equating to 10 µg of protein were diluted in 2× loading buffer and denatured at 95ºC for 5 minutes before loading into wells. Low molecular weight marker (Amersham Bioscience) was diluted according to the manufacturer's instructions and 1µl added to each of the end wells. Gels were run for 30 minutes at 70 V before increasing to 150 V until the dye front reached the bottom of the gel at which point separation was deemed complete.

2.10 Gel Visualisation – Coomassie staining

Following run completion, gels were removed from casts and sensitised in a fixative solution (40% methanol, 10% acetic acid and 50% ddH_2O) with gentle agitation for one hour. Coomassie stock solution was prepared with 0.1% Coomassie Brilliant Blue G250, 2% w/v ortho-phosphoric acid and 10% (w/v) ammonium sulphate. Stock solution was diluted (20% v/v methanol and 80% w/v stock solution) in order to create

a working solution that was applied to each gel. Gels were washed twice in ddH_2O following their removal from fixative and submerged in 40 ml of Coomassie working solution overnight. Following staining, gels were de-stained in 1% acetic acid removing background colouration leaving protein spots visible. Following staining gels were imaged using a GS-800 calibrated densitometer (Bio-Rad, UK) set to 300 dpi at 63.5-micron resolution and processed using Quantity One Version 4.6 (Bio-Rad, U.K.). Gels were stored in 2 ml of 1% acetic acid in heat-sealed bags prior to down-stream mass spectrometry analysis.

2.11 Mass Spectrometry – Trypsin Digestion

Following staining and imaging, band were manually excised from protein gels and placed into individual eppendorfs and prepared for trypsin digestion and mass spectrometry as described by Morphew *et al.,* 2011. Coomassie stained gel bands were excised and destained in 50 mM ammonium bicarbonate (AMBIC) (pH 8.0) and 50% v/v acetonitrile (ACN) for 15 minutes at 37^{o}C. This de-staining was repeated until blue colouration was completely removed. Following destaining bands were dehydrated in 100% ACN for 15 minutes at 37^{o}C and subsequently dried at 50^{o}C with their lids open. Gel pieces were then rehydrated with 50 mM AMBIC containing 10 mM DTT and incubated at 80^{o}C for 30 minutes, supernatants were removed and 50 mM AMBIC containing 55 mM IAA added and incubated at room temperature for 20 minutes in the dark. The supernatants were again removed and two washes with 50% v/v 50 mM AMBIC and 50% v/v ACN each incubated for 15 minutes at room temperature. Gel pieces were then dehydrated in 100% ACN for 15 minutes and again dried at 50^{o}C with the lids open. Gel pieces were rehydrated using 50 mM AMBIC containing 10 ng/µl Promega grade trypsin and incubated over night at 37^{o}C.

Following incubation samples were briefly centrifuged and 20-50 µl ddH_2O added to each sample and agitated at room temperature for 10 minutes. The resulting supernatants were removed and stored and 50% v/v ACN, 5% v/v formic acid and 45% ultrapure milliQ water added to the gel pieces, and subject to shaking for 1 hour at room temperature. Supernatants were again removed and added to the previously removed sample. These supernatants were subject to drying in a speed vacuum and the resulting pellet stored at -20^{o}C. Pellets were defrosted and resuspended in 20 µl of 0.1% formic acid prior to mass spectrometry analysis.

2.12 Mass Spectrometry – Electrospray ionisation quadrupole time-of-flight mass spectrometry (ESI-QUAD-TOF)

Trypsin digested samples were analysed using a liquid chromatography tandem mass spectrometer (Agilent 6550 iFunnel Q-Tof) combined with a HPLC-Chip (1200 series, Agilent Technologies, U.K.). Each sample was injected into an enrichment column within the system at a flow rate of 2.5 µl/min using an automated micro sampler with an injection volume of 2 µl in the resuspension buffer 0.1% v/v formic acid and allowed to separate at 300 nl/min. Enrichment and separation were carried out on a polaris chip (G4240-62030, Agilent Technologies, U.K). A system of solvents was utilised over the process, solvent A (milliQ water containing 0.1% formic acid) and solvent B (90% v/v acetonitrile containing 0.1% v/v formic acid). Chromatography was achieved using a pieve linear-gradient of 3-8% solvent B over 6 seconds, 8-35% solvent B over 15 minutes, 35-90% solvent B over five minutes and finally 90% solvent B for two minutes.

2.13 Tandem Mass Spectrometry ion searches

Following mass spectrometry, the files containing the peak spectra data were loaded onto Agilent Qualitative analysis software (Agilent technologies LDA UK Limited,

UK). Each file had compounds found by molecular feature and were saved to MGF. Mascot (www.matrixofscience.com) was used for analysis by carrying out an MS/MS ion search, settings were set for the enzyme trypsin – allowing 2 missed cleavages, with a fixed modification of carbamidomethyl (C) and a variable modification of oxidation (M) with a peptide charge of 2+, 3+ and 4+. Each sample was then searched against an in-house database composed of a transcript for *C. daubneyi*. Each of the contigs returned were then searched within an in-house copy of the transcript and the nucleotide sequence recorded. All of the contigs were then translated using ExPasy (www.expasy.com) and the sequences submitted to Blast p. The results returned were recorded including the organism from which the protein matched, as there is currently no published data for *C. daubneyi.*

CHAPTER 3.

CHARACTERISATION OF THE UNEMBRYONATED EGG AND ADULT SOLUBLE SOMATIC PROTEOMES OF *CALICOPHORON DAUBNEYI*

3.1 INTRODUCTION

Proteomic analysis is now a widely utilised functional genomic tool for discovery in many aspects of fundamental and applied biology including parasitology research (Wastling *et al.*, 2012). The term 'proteomics' was first coined by Wilkins *et al.*, 1994 and described as "The study of the full set of proteins encoded by a genome". Proteomic profiles are dynamic, with their profile dependent upon the environmental and physiological conditions at the time of analysis, with the proteins actively translated dictating the organism's biological phenotype (Barrett *et al.*, 2000). The proteome of an organism is controlled largely by differential regulation of gene expression (as studied by transcriptomic tools) and post-translation modifications (Reamtong, 2013). An organism's genome consists of all the possible proteomes regardless of the current proteome defined by the proteins actively synthesised at the time of collection. Developments in proteomics have made it an invaluable tool in molecular and cellular biology allowing the comprehensive study of complex samples (Aebersold & Mann, 2003).

Proteomic investigations allow not only identification of proteins and their abundance, but also in-depth analysis into their modifications with 2DE gel-based proteomic studies allowing resolution of up to 2000 proteins and subsequent mass spectrometry delivering assignment of functionality through analysis of produced spectra (**Figure 3.1**) (Barrett, 2009). Recent advances in transcriptome and genome studies represent an invaluable addition to proteomic studies allowing investigation into fundamental biology including potential drug and vaccine candidates (Sotillo *et al.*, 2016). These advances have been utilised in the study of many helminth parasites providing insights

into host-parasite interactions and immunopathology during infection (Sotillo *et al.*, 2019; Selkirk *et al.*, 2018; Liu *et al.*, 2007).

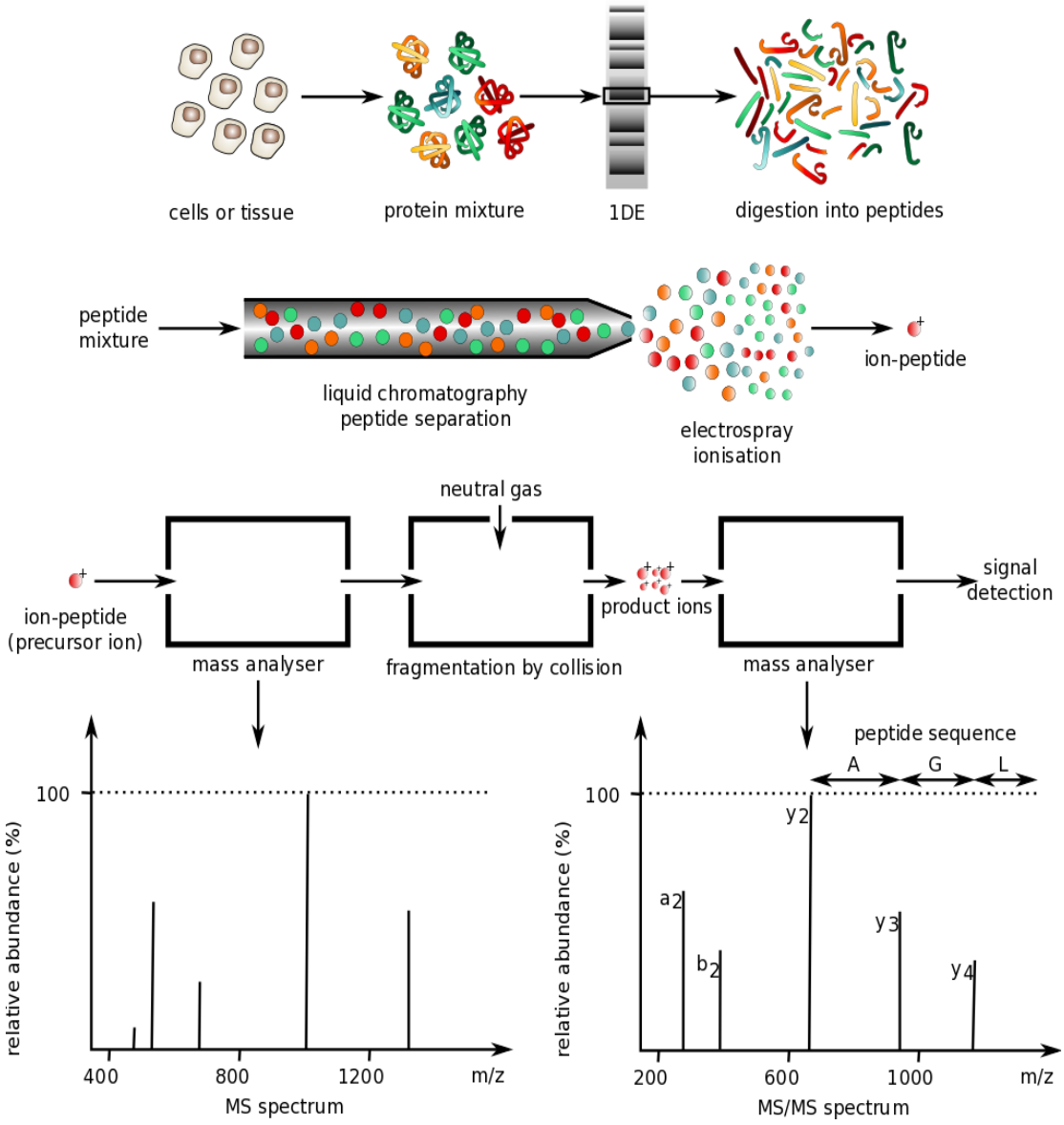


Figure 3.1: Cells or tissue are prepared into a sample containing a mixture of all the proteins present. Proteins are visualised on a polyacrylamide gel before excision and digestion into their peptide constituents. Peptides are loaded into the mass spectrometer and ionised. Ionised peptides are then sprayed and hit the 'mass analyser' at different points leading to the production of spectra comparing relative abundance to m/z value. This data can then be used in order to deduce the sequences from which the peptides originated and also allow identification of the proteins from which they originated (Emmanuel *et al.*, 2012)

3.1.1 Proteomics in understanding establishment of parasitic helminths

Helminths definitive residency within their host's immunologically-exposed environments has led to development of immune evasion mechanisms and novel parasite-host communication strategies (Johnston *et al.*, 2009; Hewitson *et al.*, 2009). Modulation of the host immune response has been attributed to parasite-derived immunomodulatory molecules, with proteins found to play a vital role (McNeilly *et al.*, 2014). Developments in 'omics' technologies have allowed the investigation of helminth parasites at a molecular level, with combined transcriptomic and proteomic studies enabling elucidation of mechanisms of host-interaction, immune system evasion, metabolism as well as identifying prospective drug targets (Tsai *et al.*, 2019; Loukas *et al.*, 2011). Proteomics, in particular, has been utilised as a powerful tool allowing resolution of helminth fundamental biology, mode of infection and pathology which are of great importance in understanding their ability to mimic/manipulate the host immune system (McKay, 2009; Mutapi, 2012).

As the agents responsible for the determination of an organism's biological phenotype, proteomic studies of helminth species have become increasingly important as they represent the target for many anthelmintic drugs as well as potential markers in the diagnosis of infection (Ndao, 2009; Barrett *et al.*, 2000). Proteomic studies of helminths have traditionally taken two approaches, the first focusing on global protein analysis of specific tissues and the second focused on the investigation of single proteins and their interactions with other proteins (Barrett, 2009). Proteomics is an essential tool in the development of new drug and vaccine targets as it allows identification of post-translational modifications that are not able to be identified through genomic and transcriptomic studies (Barrett *et al.*, 2005). As

chemotherapeutic options are currently the only effective treatment option given the observed significant development in resistance to these drugs, proteomic studies have become pivotal in the discovery and development of future treatment options (Taylor, 2013). Extensive research has been carried out on helminth Excretory/Secretory (ES) proteins in the search for potential diagnostic markers and drug targets due to their plethora of immunomodulatory molecules (Dalton *et al.,* 2003). Specific protein families such as fatty acid binding proteins (FABPs), cathepsins and glutathione transferases (GSTs) have been identified as potential drug targets in numerous helminth species due to their confirmed immunomodulatory action with studies in *Schistosoma mansoni* highlighting GSTs as promising drug targets in pre-clinical trials (Santini-Olivera *et al.,* 2016; Riveau *et al.,* 2012).

The understudied eggs of many helminth parasites could represent a future avenue of research in immunodiagnostics and drug design (Moxon *et al.,* 2008; 2010). Previous studies into egg proteins have provided important information on immune modulation and pathology in helminth species (Cass *et al.,* 2007), with studies of *S. mansoni* showing eggs to have a direct role in observed pathology associated with infection (DeMarco *et al.,* 2009). Studies have identified multiple egg proteins as potential diagnostic markers of infections, with studies of *F. hepatica* identifying members of the superfamily alpha-crystallin containing small heat shock proteins (AC/sHSP) to be promising as a diagnostic (Moxon *et al.,* 2010). Helminth eggs play a vital role in the diagnosis of helminth infections with faecal egg counts (FEC) the long-standing 'gold-standard' of diagnosis and are currently utilised in the identification of infection with *C. daubneyi* (Malrait *et al.,* 2015). FEC is a time-consuming method and is difficult in practice due to the similarity in egg morphology between trematode

species, namely *F. hepatica* (Foster *et al.,* 2008). These similarities alongside increased prevalence of *C. daubneyi* infections and co-infections with *F. hepatica* is likely to lead to misdiagnosis of infection through false positives and subsequent treatment failure, in turn, contributing to the rise of anthelmintic resistance (Rojo-Vazquez *et al.,* 2012; Gordon *et al.,* 2013; Mason *et al.,* 2012). Due to the problematic nature of FEC in identifying paramphistome infections a number of serological and molecular methods have been developed for helminth infection diagnosis (Sirisinha *et al.,* 1991; Wongratanacheewin *et al.,* 2002), with specific research on coproantigen detection methods following acknowledgement of priority by the WHO disease reference group on helminths (McCarthy *et al.,* 2012). Preliminary studies into the use of coproantigen ELISAs (cELISA) as a means of diagnosis appear to accurately distinguish between rumen and liver fluke in co-infected hosts, thus allowing administration of correct treatment options and have been found to be more sensitive than traditional FEC methods (Teimoori *et al.,* 2016; Gordon *et al.,* 2013). The overlap and co-infecting nature of these two parasites are problematic in livestock and so this is the first in-depth proteomic study into *C. daubneyi* eggs and a starting point for potential differentiation of the two through investigation into possible biomarkers unique to *C. daubneyi*.

Thus, as described above, the unique power of proteomics in investigating the fundamental biology of organisms at the protein level can be exploited in parasite studies allowing discovery of markers of infections and potential drug and vaccine candidates. Thus, this chapter will attempt to resolve the proteomic profile of *C. daubneyi* soluble somatic and egg fractions. As with many previous studies a gel-based proteomic approach was first utilised to visualise their proteomic profile.

Moreover, due to the importance of transcriptomics in elucidating functional biology of parasites a combined approach has been used herein the investigation *C. daubneyi.*

3.1.2 CHAPTER AIMS

Therefore, the specific aims of this chapter are to:

- Visualise the egg and adult soluble somatic proteomic profiles of *C. daubneyi.*
- Identify the most abundant soluble somatic and egg proteins in adult *C. daubneyi* through interrogation of the resolved transcriptome database.
- Interrogate the resolved proteomic profiles for protein families of interest allowing insight into *C. daubneyi* fundamental biology and mechanisms utilised in immune evasion and successful establishment of infection.

3.2 METHODS

Unless stated otherwise, all methods were carried out as stated in **Chapter 2**. All solutions were made up using ddH_2O and molecular grade reagents.

3.2.1 Species Identification – DNA extraction, PCR and visualisation

Genomic DNA was extracted from fluke randomly selected following parasite maintenance using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany). All extractions were subject to species-specific PCR utilizing primers designed by Martinez-Ibeas *et al* (2013), amplifying the 885 bp region of the COX1 mtDNA gene of *C. daubneyi* thus allowing direct species identification. The cocktail undergoing PCR contained 0.5 µl DNA template, 12.5 µl MyTaq Red Taq polymerase mixture (Bioline, UK), 10 µl of nuclease-free water and 1 µl of both forward and reverse primers (from 10 µMol stocks), Cd CO1F (forward) 5'-TGGAGAGTTTGGCGTCTTTT-3', and Cd CO1R (reverse) 5'-CCATCTTCCACCTCATCTGG-3' respectively. Amplification was carried out in a polymerase chain reaction (PCR) machine (Techne TC-4000 thermal cycler, Bibby Scientific, UK) and was achieved through a 95^{o}C initial denaturation for 5 minutes followed by 35 cycles of 95^{o}C for 30 seconds, 65^{o}C for 30 seconds, 72^{o}C for 30 seconds and a final extension cycle of 10 minutes at 72^{o}C. 2 µl of each product was loaded onto a 1% agarose TAE gel, and visualised using GelRed (Biotium, Hayward, USA). Identification of a single correctly sized product indicative of successful amplification was deemed a positive identification of *C. daubneyi*.

3.3 RESULTS

3.3.1 Collection and positive identification of C. daubneyi

Parasites were successfully collected from both infected dairy and beef cattle in a local Welsh abattoir, with infection confirmed through morphology and residency within the host. Infected animals were usually found to have a high fluke burden (1000+) and rumen atrophy and papillae were observed in all infected animals. In total, 11 sample collections were carried out between November 2015 and March 2018 from a total of 23 infected bovine rumens. Random selection was utilised and samples from each collection were submitted to speciation through PCR with *C. daubneyi* specific CO1 primers. All parasites collected and subsequently utilised in these experiments were positively identified as *C. daubneyi* (**Figure 3.2**). Prior to culture parasites were washed three times in phosphate buffered saline (PBS) to remove host contaminants and each wash retained allowing collection of eggs. Microscopic investigation was carried out in order to identify the presence of unembryonated eggs before separation using a series of filters. Following positive identification of eggs in wash material, the eggs were stored allowing their use in future experiments.

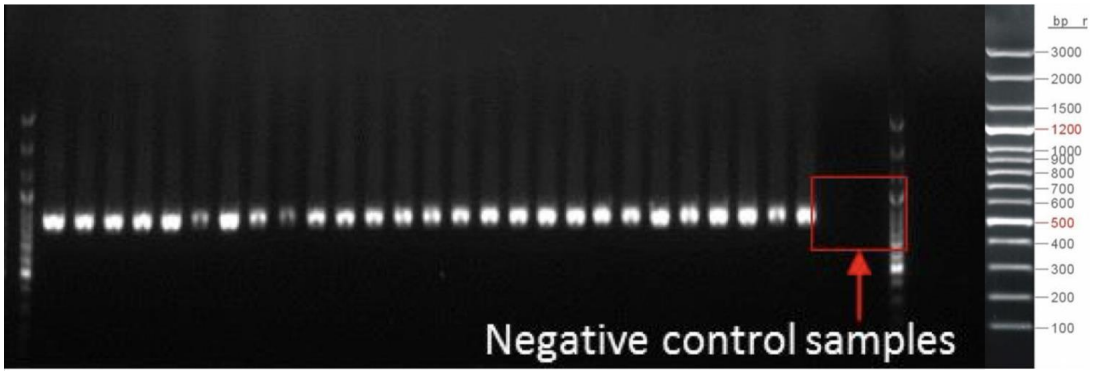


Figure 3.2: 1% TAE agarose gel of PCR products from DNA extracted and amplified with *C. daubneyi* specific CO1 primers. Positive amplification was taken as a positive ID for *C. daubneyi.*

3.3.2 Visualisation of egg and somatic soluble proteome

Homogenised adult somatic and egg samples produced consistent and reproducible 2D profiles when run on 12.5% polyacrylamide gels. Due to the large number of protein spots identified on each of the replicate gels and the complexity of cutting such a large number of spots accurately these gels were not utilised in resolution of the proteome. Instead, 1D profiles of the samples were run in triplicate and each cut into a series of 11 'bands' in order to be processed and subject to tandem mass spectrometry ensuring inclusion of all proteins present (**Figure 3.3**).

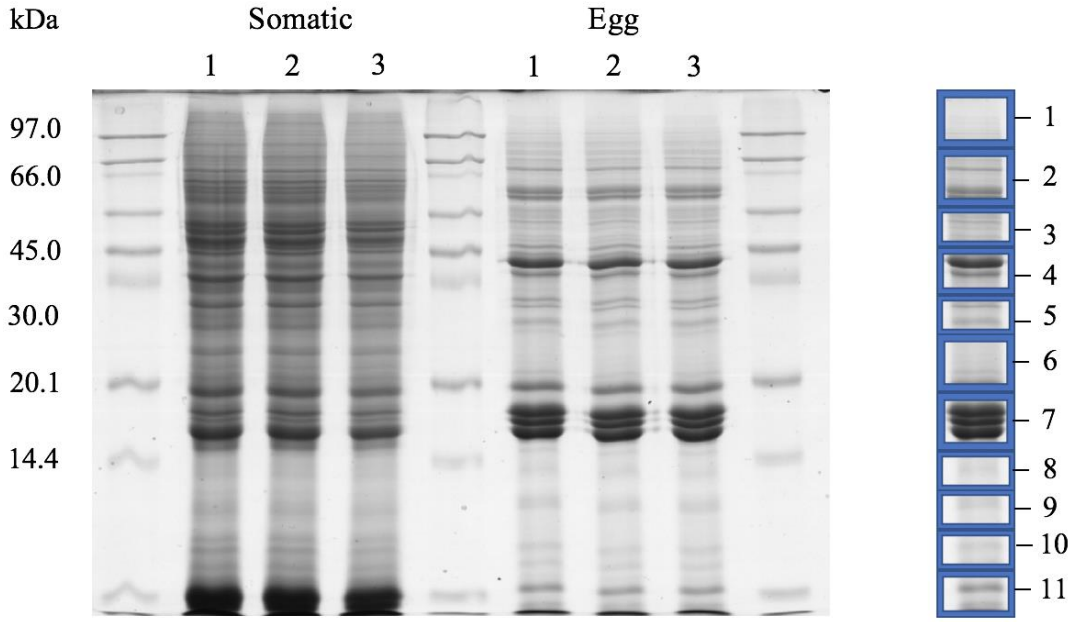


Figure 3.3: Representative 1D 7cm protein profile of adult *C. daubneyi* soluble somatic and egg fractions. 10 µg of sample was loaded for each lane and separated by 12.5% SDS-PAGE, the gel was then Coomassie Blue stained for visualisation before mass spectrometry (n=3). Representative division of lane into bands for LC-MS/MS preparation.

3.3.3 Resolution of C. daubneyi egg soluble proteome

Thus, in order to obtain the global soluble proteomic profile of *C. daubneyi* eggs via 1D each lane was divided into 11 bands consistent across each replicate (**Figure 3.3**). Each band was excised and subjected to tryptic digest followed by LC-MS/MS (**Section 2.11 – 2.13**). Returned spectra were subsequently searched against an in-house transcriptome allowing identification of proteins present within each defined band. Data from each of the 11 bands were then combined to create a single replicate of the whole proteome. Protein hits returned were ranked through the number of unique peptides associated with each protein hit and those below the significance threshold (>47) excluded from the results. Only hits replicable across all three replicates were included in the final compilation of proteins identified and duplicates sequences within replicates removed.

In total the *C. daubneyi* egg proteome returned a total of 307 proteins that were found to be consistent across replicates. When looking at each replicate individually, replicate 1 identified a total of 626 proteins, replicate 2 identified 544 proteins and replicate 3 identified 578 proteins. However, for each of these replicates proteins below the significance threshold represented 116, 110 and 128 sequences respectively. Each sequence identified was subjected to translation and subsequent BlastP analysis on the NCBIr database in order to assign their protein ID. Blast description, organism and accession numbers for the top 50 most abundant proteins are outlined in **Table 3.1.** All of the top 50 proteins were assigned to closely related helminth species with no hypothetical or uncharacterised proteins identified (**Figure 3.4**).

Table 3.1: Summary of the 50 most abundant protein identified in the egg proteome of adult *C. daubneyi*. Proteins were identified by 1D SDS-PAGE followed by LC-MS/MS. The hits listed are the top 50 proteins hit when quantified by the number of unique peptides across three replicates. In order to be regarded as significant only hits above the threshold of 47 were included.

Transcript ID	Isoform	Mascot Score	Unique peptides	Blast Description	Organism	NCBI Accession
TR24044|c0_g1	i1	1599	29	Phosphoenolpyruvate carboxykinase	*Clonorchis sinensis*	GAA49544.1
TR20530|c0_g1	i1	1526	27	Heat shock 90	*Opisthorchis viverrini*	OON14937.1
TR19966|c0_g1	i1	531	23	Elongation factor 2	*Clonorchis sinensis*	GAA28875.2
TR19392|c0_g1	i1	1298	22	T-complex 1 subunit theta	*Schistosoma mansoni*	XP_18655017.1
TR22722|c0_g1	i1	1642	22	Transketolase	*Fasciola hepatica*	PIS87475.1
TR23192|c0_g1	i1	585	22	Ribonucleoside-diphosphate reductase large subunit	*Schistosoma mansoni*	XP_18651820.1
TR17741|c0_g1	i1	2441	21	Heat shock 70	*Fasciola hepatica*	PIS90712.1
TR22802|c0_g1	i1	1204	21	T-complex 1 subunit beta	*Schistosoma japonicum*	CAX69820.1
TR24862|c0_g1	i1	286	21	Clathrin heavy chain	*Fasciola hepatica*	PIS89614.1
TR19308|c0_g1	i1	1090	20	Heat shock 60	*Schistosoma haematobium*	XP_12800213.1
TR20145|c0_g1	i1	1005	20	Major egg antigen	*Clonorchis sinensis*	GAA51199.1
TR23430|c0_g1	i1	334	19	Importin-5	*Schistosoma mansoni*	XP_18655445.1
TR16656|c0_g1	i1	845	19	T-complex 1 subunit alpha	*Scistosoma mansoni*	XP_018650657.1
TR17749|c0_g1	i1	765	19	Transitional endoplasmic reticulum ATPase	*Schistosoma mansoni*	XP_18647063.1
TR23620|c0_g1	i1	1409	18	Heat shock 70	*Fasciola hepatica*	PIS90712.1
TR25182|c1_g1	i1	575	18	Cell polarity	*Scistosoma mansoni*	XP_18652630.1
TR14742|c0_g1	i1	493	18	1,4-alpha-glucan-branching enzyme	*Schistosoma haematobium*	KGB36622.1

Table 3.1: *continued.*

TR16652|c0_g1	i1	387	18	T-complex 1 subunit gamma-like	*Opisthorchis viverrini*	OON20242.1
TR19186|c0_g1	i1	483	18	Disulfide-isomerase	*Clonorchis sinensis*	XP_9170645.1
TR17367|c0_g1	i1	1279	17	Enolase	*Fasciola hepatica*	PIS80121.1
TR25501|c0_g1	i2	123	17	Pyruvate carboxylase	*Schistosoma haematobium*	XP_12793920.1
TR20937|c0_g1	i1	1074	17	Hypothetical protein	*Opisthorchis viverrini*	KER23377.1
TR20071|c0_g1	i1	411	16	Dipeptidyl peptidase 3	*Schistosoma mansoni*	XP_18651507.1
TR22625|c1_g1	i1	148	15	Puromycin-sensitive aminopeptidase-like isoform	*Clonorchis sinensis*	GAA51710.1
TR15705|c0_g1	i1	479	15	ATP-dependent RNA helicase eIF4A	*Clonorchis sinensis*	GAA43007.2
TR20209|c0_g1	i1	660	15	T-complex 1 subunit delta	*Clonorchis sinensis*	GAA28677.1
TR22236|c1_g1	i1	1942	15	Major egg antigen (p40)	*Clonorchis sinensis*	GAA51199.1
TR22210|c0_g1	i1	323	15	Heat shock 70 (hsp70)-	*Schistosoma mansoni*	CAZ34366.1
TR18369|c0_g1	i1	392	15	T-complex 1 subunit zeta	*Clonorchis sinensis*	GAA29285.1
TR19076|c0_g1	i1	826	15	Aldehyde dehydrogenase	*Schistosoma japonicum*	CAX73522.1
TR20952|c0_g1	i1	416	15	HSP-70	*Schistosoma japonicum*	CAX73729.1
TR17779|c0_g1	i1	1538	14	Actin	*Opisthorchis viverrini*	XP_9173847.1
TR21894|c0_g1	i1	484	14	Transaldolase	*Opisthorchis viverrini*	OON16698.1
TR9216|c0_g1	i1	1443	14	14-3-3 Protein	*Opisthorchis viverrini*	OON14987.1
TR24118|c0_g1	i1	112	13	Importin-7	*Opisthorchis viverrini*	OON18634.1

Table 3.1: *continued.*

TR15860|c0_g1	i1	2509	13	Ferritin	*Fasciola hepatica*	PIS87323.1
TR20056|c0_g1	i1	1012	13	T-complex 1 subunit eta	*Opisthorchis viverrini*	OON14882.1
TR26002|c2_g1	i1	150	13	Actin	*Fasciola hepatica*	PIS86458.1
TR19513|c0_g1	i1	857	13	Eukaryotic translation elongation factor 1 alpha 2	*Opisthorchis viverrini*	OON16001.1
TR19607|c0_g2	i1	627	13	Phosphoenolpyruvate carboxykinase	*Fasciola hepatica*	PIS91518.1
TR19739|c0_g1	i1	812	13	SH3-domain GRB2-like endophilin B1	*Schistosoma mansoni*	CCD74824.1
TR21959|c0_g1	i1	586	13	Asparagine--tRNA cytoplasmic	*Schistosoma japonicum*	CAX73852.1
TR23283|c0_g1	i1	113	13	Staphylococcal nuclease domain-containing 1-like	*Clonorchis sinensis*	GAA55976.1
TR24356|c0_g1	i1	59	13	Programmed cell death 6-interacting	*Clonorchis sinensis*	GAA36539.2
TR24460|c0_g1	i1	208	13	Coatomer subunit alpha	*Schistosoma haematobium*	XP_12795585.1
TR25005|c0_g1	i1	183	13	Glycine--tRNA ligase	*Opisthorchis viverrini*	OON14524.1
TR16120|c0_g1	i1	4483	13	Soma ferritin	*Fasciola hepatica*	PIS87323.1
TR17164|c0_g1	i1	1241	13	Glyceraldehyde 3-phosphate dehydrogenase	*Clonorchis sinensis*	GAA28380.1

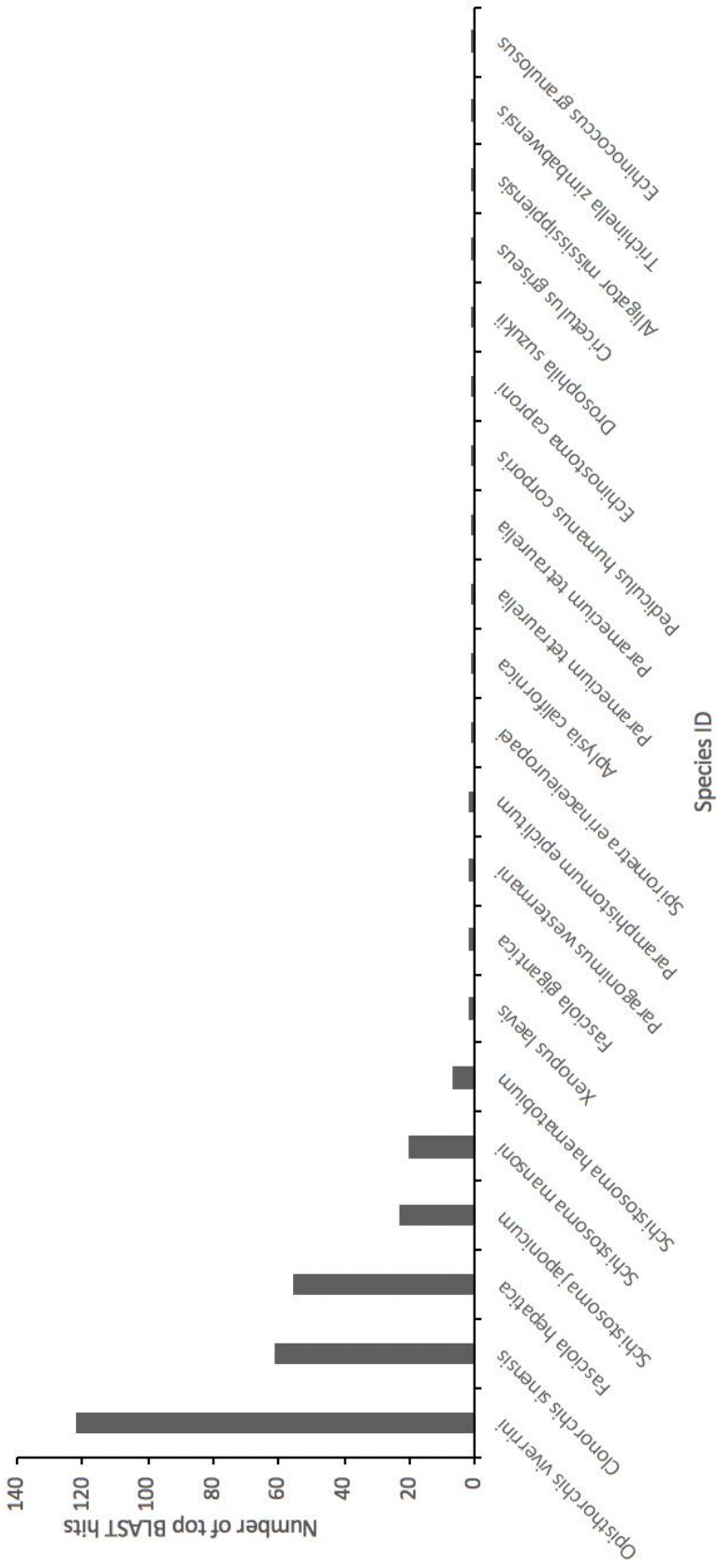


Figure 3.4: Species distribution of the top 20 species hit following BlastP analysis of the sequences returned from the *C. daubneyi* egg proteome. Species distribution analyzed using Blast2Go (n=3).

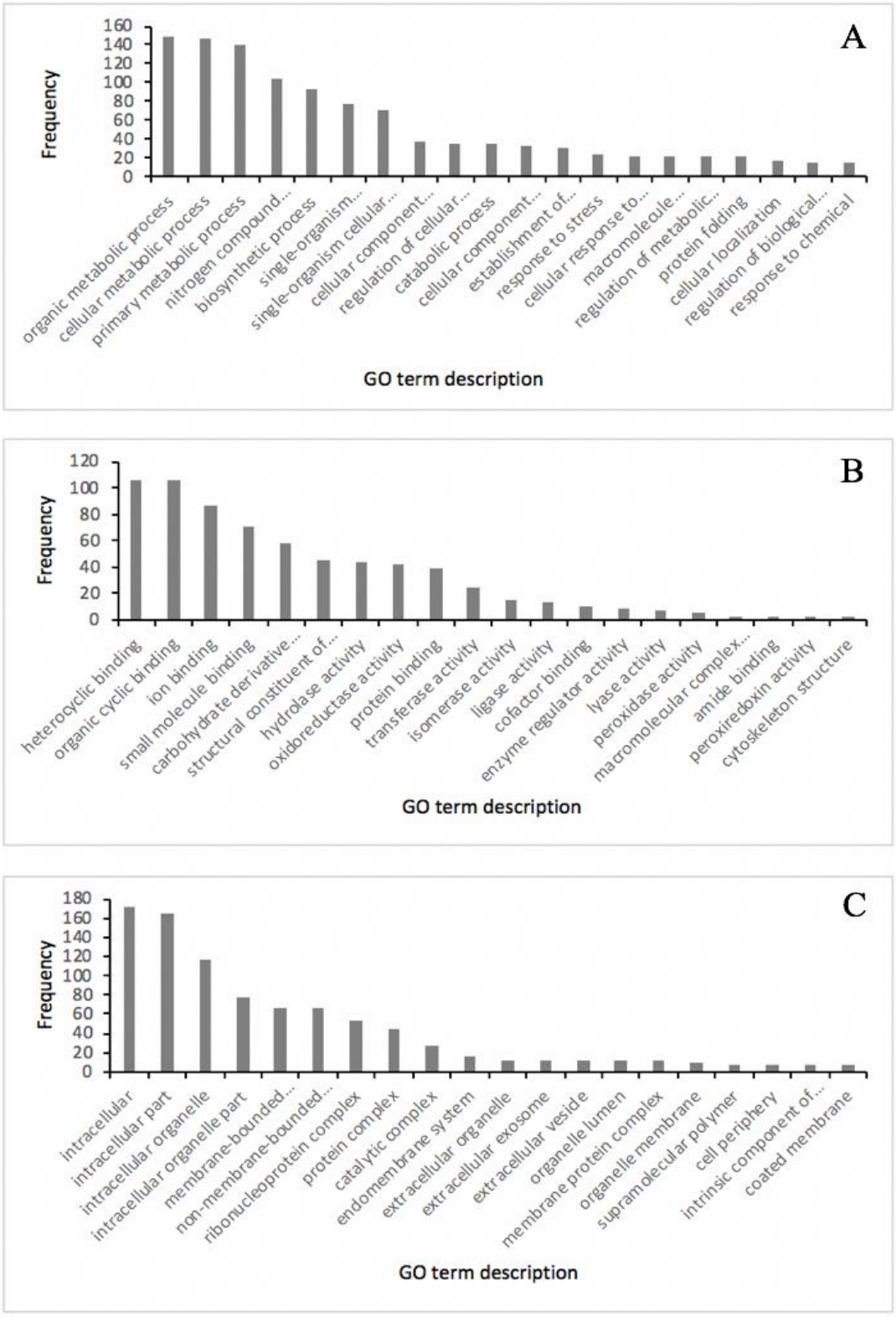


Figure 3.5: Level 3 gene ontology categories for each protein identification in *C. daubneyi* eggs. Protein sequences were assigned into 3 functional groups -
A) Molecular Feature B) Biological process and C) Cellular component. Gene ontology categories were assigned using Blast2Go (n=3).

3.3.4 Characterisation of a C. daubneyi AC/sHSP

Representatives of the AC/sHSP superfamily were identified in helminth species and retrieved from the NCBIr database. Closely related *F. hepatica* (FhHSP35α) and *S. mansoni* (Sm-p40) AC/sHSP sequences were utilised in mining of the *C. daubneyi* transcriptome allowing identification of homologous sequences (Moxon *et al.*, 2010). Local blast analysis against the in-house transcriptome resolved 16 sequences homologous to FhHSP35α and 14 sequences homologous to Sm-p40. The top 3 results (based on E-value) from both represented three isoforms of a single protein - TR21269 (c0_g1_i1, c0_g1_i2 and c0_g1_i3) (**Table 3.2 and Table 3.3**).

Retrieved sequences were subject to BLAST and Pfam analysis confirming their characterisation as AC/sHSP's with top blast results matched to experimentally validated sequences and domains specific to the superfamily. Resolved sequences were searched in the resolved egg proteome identifying 3 to be present, TR21269|c0_g1_i1, TR20145|c0_g1_i1 and TR22236|c1_g1_i1. The sequence with the most significant sequence similarity was subject to ClustalW alignment with seven representative helminth AC/sHSP sequences identifying multiple conserved regions as well as areas of physiochemical similarity (**Figure 3.6**) further confirming the identification of members of the superfamily AC/sHSP in *C. daubneyi*.

Table 3.2: *C. daubneyi* transcripts identified to have significant similarity to *Schistosoma* Sm-p40. In total 16 homologous sequences were identified and their presence in the resolved egg proteome noted. Alternate shading shows different isoforms identified.

Sequences with significant alignment	Score (bits)	E value	Identity in Proteome
TR21269|c0_g1_i2	268	5.00E-72	
TR21269|c0_g1_i1	268	8.00E-72	Yes
TR21269|c0_g1_i3	267	1.00E-71	
TR21269|c0_g1_i4	95	9.00E-20	
TR18775|c0_g1_i1	241	6.00E-64	
TR18775|c0_g1_i2	139	4.00E-33	
TR18141|c0_g1_i4	58	2.00E-08	
TR18141|c0_g1_i3	55	8.00E-08	
TR18141|c0_g1_i2	54	2.00E-07	
TR15958|c0_g2_i1	84	2.00E-16	
TR15958|c1_g1_i1	121	9.00E-28	
TR21716|c0_g1_i1	229	3.00E-60	
TR20145|c0_g1_i1	224	8.00E-59	Yes
TR22236|c1_g1_i1	218	7.00E-57	Yes
TR22236|c1_g2_i1	76	4.00E-14	
TR19516|c0_g1_i1	111	1.00E-24	

Table 3.3: *C. daubneyi* transcripts identified to have significant similarity to *Fasciola* FhHSP35α. In total 14 homologous sequences were identified and their presence in the resolved egg proteome noted. Alternate shading shows different isoforms identified.

Sequences with significant alignment	Score (bits)	E value	Identity in Proteome
TR21269|c0_g1_i2	229	3.00E-60	
TR21269|c0_g1_i1	228	8.00E-60	Yes
TR21269|c0_g1_i3	225	5.00E-59	
TR21269|c0_g1_i4	92	5.00E-19	
TR18775|c0_g1_i1	216	2.00E-56	
TR18775|c0_g1_i2	120	2.00E-27	
TR15958|c1_g1_i1	92	7.00E-19	
TR15958|c0_g2_i1	72	9.00E-13	
TR22236|c1_g2_i1	79	4.00E-15	
TR22236|c1_g1_i1	198	7.00E-51	Yes
TR20145|c0_g1_i1	209	3.00E-54	Yes
TR21716|c0_g1_i1	178	7.00E-45	
TR19516|c0_g1_i1	99	5.00E-21	
TR18141|c0_g1_i4	52	6.00E-07	

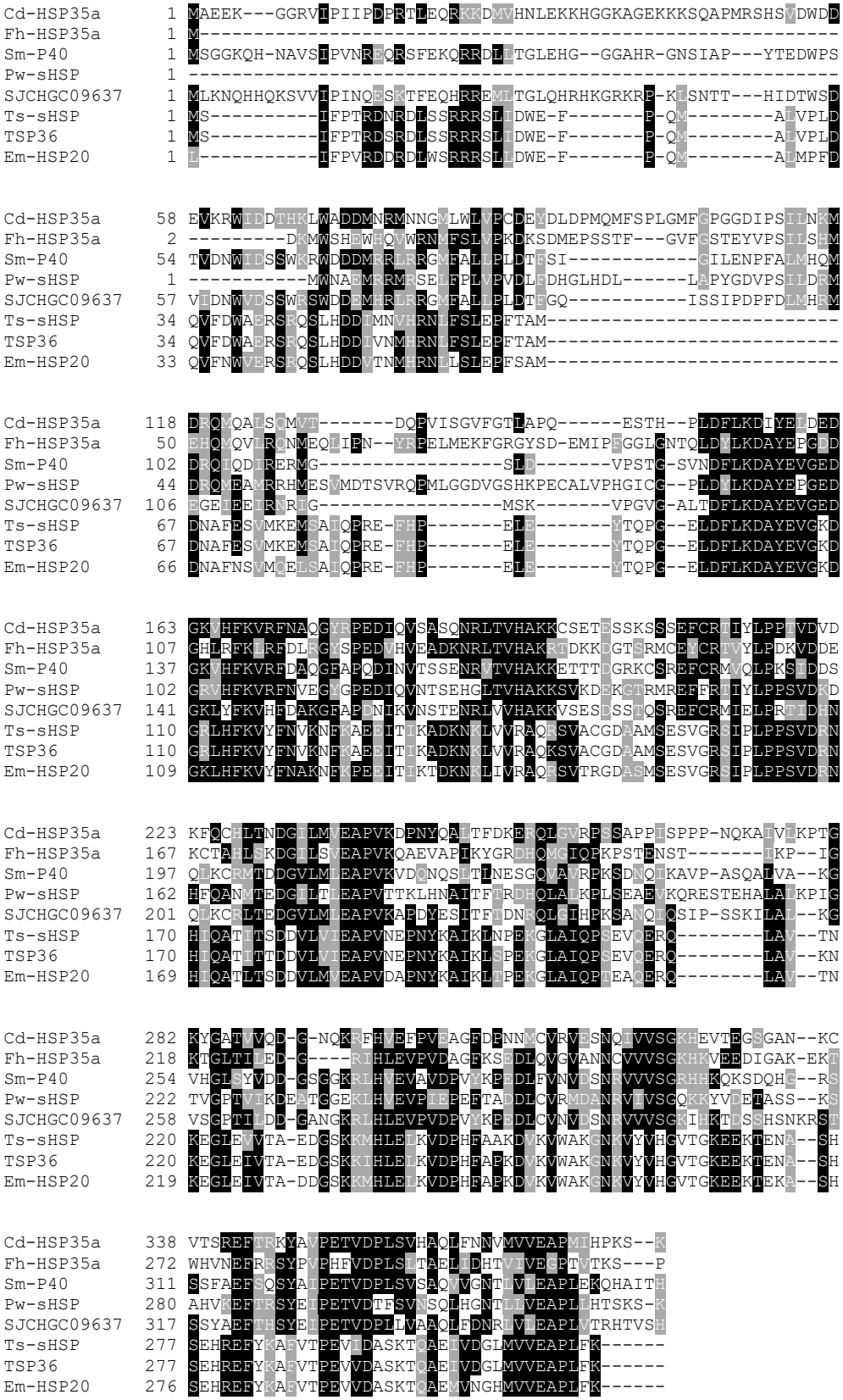


Figure 3.6: Multiple sequence alignment of *C. daubneyi* sequence TR21269|c0_g1_i1 (Cd-HSP35α) and orthologues expressed by *S. mansoni* (Sm-P40 accession P12812), *S. japonicum* (SJCHGC09637 accession AAW25328), *P. westermani* (Pw-sHSP accession AAK35217), *Taenia solium* (Ts-sHSP accession CAD36617), *T. saginata* (TSP36 accession Q7YZT0), and *Echinococcus multilocularis* (Em-HSP20 accession CAD12371). Conserved amino acids across all sequences are highlighted in black and amino acids of physiochemical similarity are highlighted in grey.

3.3.5 Resolution of C. daubneyi soluble somatic proteome

As with the resolution of the *C. daubneyi* egg proteome, the soluble somatic proteome was resolved using 1D SDS-PAGE prior to LC-MS/MS analysis. In total the *C. daubneyi* soluble somatic proteome returned a total of 658 sequences consistent across all 3 replicates above the significance threshold of 47. The sequences consistent across replicates were again sorted by the number of unique peptides allowing resolution of the most abundant proteins. Sequences returned were subject to translation followed by BlastP analysis the top 50 of which are outlined in **Table 3.4.**

Interestingly, the soluble somatic proteome returned a high number of proteins that did not return any matches when subject to blast analysis as well as returning a high number of hypothetical and uncharacterized proteins. These proteins could represent those unique to *C. daubneyi* that are utilized in the successful establishment of infection. In total these proteins accounted for 32% of the top 50 most abundant proteins in the *C. daubneyi* soluble somatic proteome including the three most abundant proteins, TR25975|c0_g1, TR26102|c0_g1 and TR25969|c0_g1, with subsequent Pfam and Interpro analysis identifying no conserved domains that could elucidate their function.

Proteins resolved in the *C. daubneyi* soluble somatic proteome were subsequently analysed for their species distribution (**Figure 3.7**) and GO classifications (**Figure 3.8**), allowing elucidation of the species to which they shared significant sequence similarity and assignment of their function.

Table 3.4: Summary of the 50 most abundant protein identified in the soluble somatic proteome of adult *C. daubneyi*. Proteins were identified by 1D SDS-PAGE followed by LC-MS/MS. The hits listed are the top 50 proteins hit when quantified by the number of unique peptides across three replicates. In order to be regarded as significant only hits above the threshold of 47 were included.

Transcript ID	Isoform	Mascot Score	Unique peptides	Blast Description	Organism	NCBI Accession
TR25975|c0_g1	i1	2592	110	-	-	-
TR26102|c0_g1	i1	678	60	-	-	-
TR25969|c0_g1	i1	1854	56	-	-	-
TR24746|c0_g1	i2	2176	48	Myosin	*Clonorchis sinensis*	GAA54503.1
TR24694|c0_g1	i1	741	40	Spectrin	*Opisthorchis viverrini*	XP_9165214.1
TR25501|c0_g1	i2	1801	38	Pyruvate carboxylase	*Schistosoma haematobium*	XP_12793920.1
TR25376|c0_g1	i1	550	35	Spectrin	*Opisthorchis viverrini*	OON21331.1
TR25928|c0_g1	i1	982	33	-	-	-
TR23608|c0_g1	i1	2506	30	Propionyl-CoA carboxylase	*Clonorchis sinensis*	GAA57227.1
TR23048|c0_g1	i2	1274	30	Glycogen phosphorylase	*Schistosoma haematobium*	KGB33031.1
TR23603|c0_g2	i2	657	30	Myosin	*Fasciola hepatica*	PIS89181.1
TR24885|c1_g1	i1	2260	27	Aminoadipicsemialdehyde synthase	*Clonorchis sinensis*	GAA52140.1
TR20530|c0_g1	i1	3188	26	Heat shock 90	*Opisthorchis viverrini*	OON14937.1
TR24485|c0_g1	i1	935	26	Myosin tail	*Fasciola hepatica*	PIS89678.1
TR25871|c0_g2	i1	907	25	Filamin	*Echinococcus multilocularis*	CDS40988.2
TR17749|c0_g1	i1	1423	22	Cell division control	*Opisthorchis viverrini*	OON16244.1

Table 3.4: *Continued.*

TR25960|c0_g1	i1	464	22	a-macroglobulin	*Opisthorchis viverrini*	OON18030.1
TR17741|c0_g1	i1	2380	22	Heat shock 70	*Fasciola hepatica*	PIS90712.1
TR21565|c1_g1	i1	7580	21	Aldehyde dehydrogenase	*Schistosoma japonicum*	CAX73522.1
TR19392|c0_g1	i1	1986	21	Chaperonin	*Schistosoma mansoni*	XP_18655017.1
TR20947|c0_g2	i1	952	21	Major vault protein	*Schistosoma mansoni*	XP_18648228.1
TR25407|c0_g1	i1	254	21	-	-	-
TR22802|c0_g1	i1	1551	20	Chaperonin containing t-complex	*Schistosoma japonicum*	CAX69820.1
TR21551|c0_g1	i1	657	20	-	-	-
TR24693|c0_g1	i1	719	20	Urocanate hydratase	*Opisthorchis viverrini*	OON23076.1
TR24862|c0_g1	i1	249	20	-	-	-
TR23114|c0_g2	i1	1129	20	Hypothetical protein	*Opisthorchis viverrini*	XP_9170800.1
TR23620|c0_g1	i1	1851	19	Heat shock 70	*Fasciola hepatica*	PIS90712.1
TR22712|c0_g1	i1	1305	19	Dihydrolipoamide dehydrogenase	*Schistosoma mansoni*	XP_18650285.1
TR19268|c0_g1	i1	1655	19	Tegument Antigen	*Clonorchis sinensis*	GAA49983.1
TR19607|c0_g2	i1	1644	19	Phosphoenolpyruvate carboxykinase	*Fasciola hepatica*	PIS91518.1
TR19308|c0_g1	i1	1148	19	Heat shock 60	*Schistosoma haematobium*	XP_12800213.1
TR24050|c0_g1	i1	1090	19	Calpain	*Schistosoma haematobium*	BAF62290.1
TR23080|c0_g1	i1	430	19	-	-	-

Table 3.4: *Continued.*

TR19458|c0_g1	i1	1389	19	Glutamate dehydrogenase	*Clonorchis sinensis*	GAA53751.1
TR25027|c0_g1	i1	602	19	-	-	-
TR16656|c0_g1	i1	748	18	-	-	-
TR25567|c0_g1	i1	362	18	-	-	-
TR18389|c0_g1	i1	1102	18	Citrate synthase	*Schistosoma haematobium*	XP_12795827.1
TR17690|c0_g1	i1	2416	18	Propionyl-CoA carboxylase	-	GAA51736.1
TR23286|c0_g1	i1	1366	18	Leukotriene hydrolase	*Clonorchis sinensis*	GAA49617.1
TR16652|c0_g1	i1	551	18	-	-	-
TR20937|c0_g1	i1	1523	18	Hypothetical protein	*Opisthorchis viverrini*	KER23377.1
TR12762|c0_g1	i1	1835	17	Succinate CoA-transferase	*Fasciola hepatica*	ACF06126.1
TR19186|c0_g1	i1	853	17	Disulfide-isomerase	*Opisthorchis viverrini*	XP_9170645.1
TR22625|c1_g1	i1	458	17	-	-	-
TR19883|c4_g1	i1	615	17	LIM domain	*Opisthorchis viverrini*	OON22809.1
TR17099|c2_g1	i1	1171	17	Tubulin beta	*Clonorchis sinensis*	GAA51682.1
TR16503|c0_g1	i1	935	17	Aspartate aminotransferase	*Schistosoma mansoni*	CCD77440.1
TR23254|c0_g1	i1	485	16	-	-	-

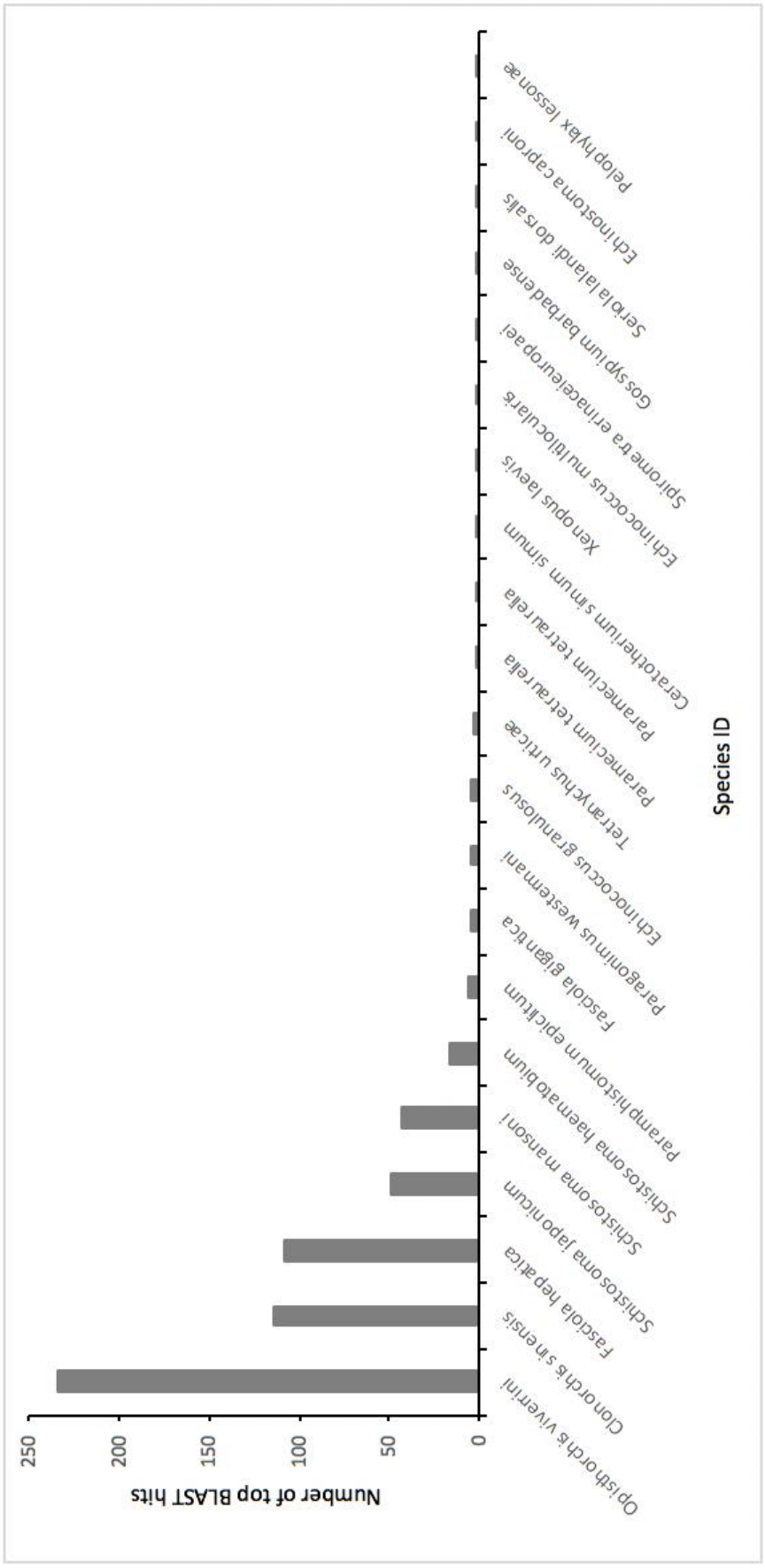


Figure 3.7: Species distribution of the top 20 species hit following BlastP analysis of the sequences returned from the *C. daubneyi* soluble somatic proteome. Species distribution analyzed using Blast2Go (n=3).

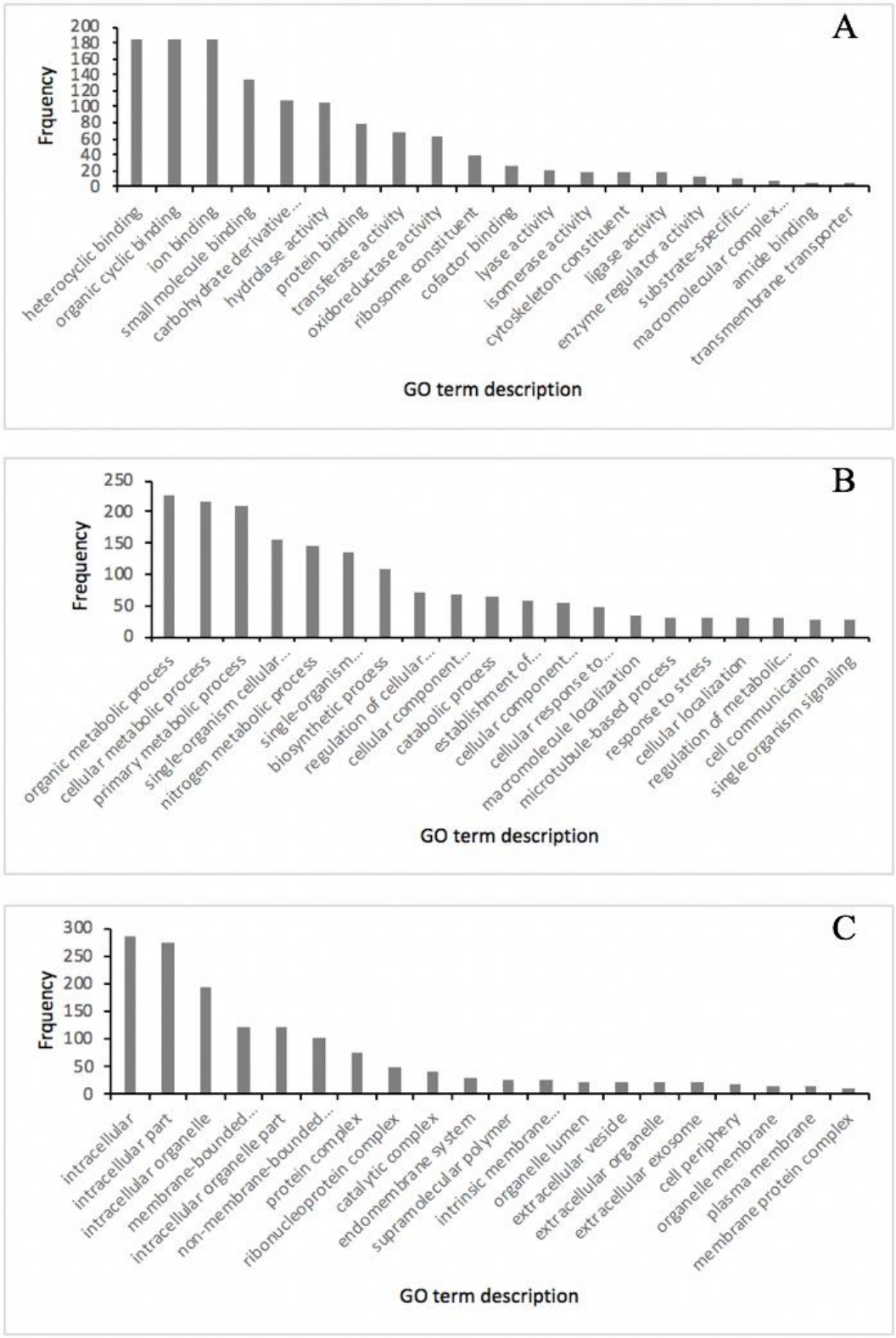


Figure 3.8: Level 3 gene ontology categories for each protein identification in *C. daubneyi* eggs. Protein sequences were assigned into 3 functional groups
A) Molecular Feature B) Biological process and C) Cellular component. Gene ontology groups were assigned using Blast2Go (n=3).

3.4 DISCUSSION

Despite their animal welfare and economic importance, veterinary helminth parasites remain largely understudied due to their complexity (Hotez, 2009), however, developments in -omic technologies are now allowing their study at a molecular level allowing elucidation of their basic biology, infection establishment and potential pathological effects (Loukas *et al.*, 2011; Mutapi, 2012). Studies suggest that proteins represent some of the key molecules responsible for immune modulation, and utilisation of the newly published transcriptome allowed for the first time, a comprehensive proteomic analysis of *C. daubneyi* adult worms and eggs allowing insight into the parasite's life cycle, mechanisms of immune evasion and successful establishment within the host (Huson *et al.*, 2017; Johnston *et al.*, 2009).

Currently *C. daubneyi* represents the only rumen fluke species found to infect ruminant livestock in the UK with studies suggesting it may be the only species found in Western Europe (Malrait *et al.*, 2015). In this study naturally infected cattle were utilised for fluke collection due to increased prevalence of notable infection when compared with sheep (Toolan *et al.*, 2015). Although *C. daubneyi* is suggested as the only species in Western Europe, a single study has suggested mixed paramphistome infections in Ireland, whilst many studies failed to note the species of paramphistome being utilised (Martinez-Ibeaz *et al.*, 2016; Pavan *et al.*, 2014). It is important to note that increased prevalence of *C. daubneyi* in Western Europe has not been officially confirmed and could be due to increased awareness of the parasite leading to greater reporting of infection. The high level of livestock movement worldwide poses a threat to biosecurity and increases the risk of introduction of new paramphistome species to the U.K, with studies hypothesising that livestock movement lead originally to the

introduction of *C. daubneyi* to the UK (Perry *et al.*, 2011; Zintl *et al.*, 2014). Due to the potential risk of new species introduction it was important to resolve the specific species of paramphistome at each collection, and so the samples collected were subject to PCR speciation using species specific CO1 primers leading to positive identification of all samples used in this study as *C. daubneyi*.

When looking at the soluble somatic and egg proteome of *C. daubneyi*, a large number of proteins identified were assigned to closely related helminths and numerous invertebrate species. Analysis of 'top-hit' species distribution identified the proteins resolved in the egg proteome to belong to a total of 21 species. The top five species returned accounted for 282 out of the 308 proteins identified and were from closely related parasitic flatworm species - *Opisthorchis viverrini, Clonorchis sinensis, Fasciola hepatica, Schistosoma japonicum* and *Schistosoma mansoni*. Species distribution from the soluble somatic proteome resolved hits from 55 species of which the top 5 were accounted for by *Opisthorchis viverrini, Clonorchis sinensis, Fasciola hepatica, Schistosoma japonicum* and *Schistosoma mansoni* representing 86.2% of the sequences identified. Notably, a large portion of the proteins identified were uncharacterised/hypothetical, a high percentage of which were observed in the soluble somatic fraction. Proteins may return as hypothetical/uncharacterised as they were either previously discovered in other species with no experimental information to elucidate their functionality or they could be unique to *C. daubneyi*. The high number of these proteins observed is likely due to the lack of research/resolved genomes in many helminths due to their status as low veterinary importance. The difference in levels of uncharacterised proteins between the egg and soluble somatic fractions is

possibly due to the adult worm requiring specific proteins allowing its establishment within the host that differ to those of helminths that reside in different tissues.

1.4.1 *Proteome analysis*

Egg proteins are of particular importance in many trematode species, including schistosomes where research has concluded its pathology is due to the recognition of antigens present on the eggs surface leading to propagation of an acute inflammatory response (Pearce & MacDonald, 2002). Response to these egg antigens not only contributes to pathology but also accommodates egg migration through intestine facilitating an essential role in the maintenance of the life cycle (Doenhoff, 1997). Due to specific egg proteins playing a crucial role in the success of schistosome life cycle, *C. daubneyi* egg proteome has here been resolved. Previous investigations of the *S. mansoni* and *F. hepatica* egg proteomes identified a total of 188 proteins and 208 proteins respectively (Cass *et al*., 2007; Moxon *et al.*, 2010), whilst this study elucidated a profile of 308 proteins for *C. daubneyi* (n=3).

In total, the sequences identified in the egg proteome were assigned to Gene Ontology (GO) classes for molecular function 703 times. Many sequences were assigned to more than one category due to their homology with more than one protein family and identification of multiple conserved domains. Analysis of molecular function showed 69.2% of proteins to exhibit binding activity, accounted for by 12 binding categories including – heterocyclic (GO:1901363), organic cyclic (GO:0097159), ion (GO:0043167), small molecule (GO:0036094), carbohydrate (GO:0097367) and protein (GO:0005515). Further investigation into molecular function revealed 22.8% of the sequences to exhibit catalytic activity with hydrolase activity (GO:0016787),

oxidoreductase activity (GO:0016491) and transferase activity (GO:0016740) particularly enriched. For biological process, a total of 1336 matches to GO categories were identified, again with many sequences being assigned to more than one functional group. Physiological processes accounted for 75.1% of the assigned GO categories, with 53% attributed to organic (GO:0071704), cellular (GO:0044237), primary (GO:0044238), nitrogen (GO:0006807), biosynthetic (GO:0009058) and single organism (GO:0044710) metabolic processes. Lastly, sequences were assigned to 969 cellular components GO categories of which 54.7% were attributed to intracellular parts and organelles with a large portion of the hits accounted for by cytoplasmic organelles (GO:0043226) and protein complexes (GO:0043234). Interestingly, extracellular organelle, exosome and vesicle GO classifications were also assigned to numerous sequences which are further investigated in **Chapter 4**.

Investigation of the protein sequences returned from BlastP analysis highlighted the egg proteome contains a high number of hits to ferritin, two of which appeared in the top 50 most abundant proteins (TR15860|c0_g1 and TR16120|c0_g1). An abundance of ferritin has previously been described in helminth proteomic profiles with sequences found to be homologous with that of vertebrates and invertebrates as well as phylogenetic analyses demonstrating common ancestry between ferritin identified in many helminth species. Studies have described a strong reactivity of ferritin to *F. hepatica* sera suggesting its potential use as a diagnostic marker for fasciolosis which could also be utilised in the diagnosis of paramphistomosis (Caban-Hernandez *et al.*, 2011). Whilst high levels of ferritin have been described in many helminths, ferritin has not previously been noted in studies of *S. mansoni*, however this is likely due to the similarities between *F. hepatica* and *C. daubneyi* life-cycles and shared

intermediate host, whilst *S. mansoni'*s life cycle is vastly different with egg development occurring within the definitive host rather than in an external environment (Curwen *et al.,* 2004; Cass *et al.,* 2007).

Heat shock proteins were also found to be highly represented and have previously been described in the protection of helminths such as *S. mansoni* acting as a means of protection during the molluscan stage of their life-cycle (Wu *et al.,* 2009). Of particular interest was the identification of sequences homologous with the alpha-crystallin containing small heat shock protein previously identified in both *F. hepatica* and *S. mansoni* (FhHSP35α and Sm-p40), through local blast analysis (Moxon *et al.*, 2010). In total, 16 sequences were identified to have significant similarity ($e < 0$) with AC/sHSP's corresponding to 8 proteins following removal of isoforms. These sequences were searched against the resolved egg proteome and a total of 3 proteins were identified to be present. The top hit with the most significant sequence similarity (TR21269|c0_g1_i1) was utilised in an alignment with 7 resolved ACs/HSP proteins from helminth species showing significant similarity allowing characterisation of this sequence as a member of the ACs/HSP superfamily, representing the first identification of this superfamily in any rumen fluke species. Previous identification of FhHSP35α in *F. hepatica* was postulated as a potential diagnostic marker as the first helminth to identified to contain a representative of this superfamily. However, the identification of HSP35α in *C. daubneyi* could discredit its use as a potential biomarker in *F. hepatica* due to the rise in co-infections identified in the UK, utilisation of this protein could lead to false positives when identifying fluke infections. Identification of sHSP's in *C. daubneyi* is of particular note due to their function as molecular chaperones and in development of protection following

exposure to stress stimuli such as cold, oxidation and heavy metals, allowing hypothesis that sHSP could be responsible for successful establishment of large *C. daubneyi* burdens in the traditionally harsh rumen environment (Li *et al.*, 2009; Caspers *et al.*, 1995).

In total the proteins identified in the soluble somatic proteome were assigned to 36 GO categories associated with molecular function. Similar to the egg profile, binding activity was described in 71% of the sequences identified. In total 16 GO terms associated with binding were resolved with the top 5 identical to that of the egg profile. The 4 terms associated with the global somatic proteome that did not appear in the egg were, oxygen binding (GO:0019825), carbohydrate binding (GO:0030246), modified amino acid binding (GO:0072341) and metal cluster binding (GO:0051540). However, these only accounted for 0.6% of the total sequences resolved. In comparison 23.1% somatic GO terms were associated with catalytic activity across 15 GO identifiers, whilst the egg proteome only consisted of 13. The top proteins enriched in this experiment were the same as the egg profile however, three GO terms were unique to the egg proteome and five to the somatic. Biological processe analysis revealed a total of 2359 GO associations across 121 GO terms. Of these 67.3% of the sequences hit were associated with physiological processes, 46.1% of these accounted for by the top six GO terms which were the same as those identified in the egg proteome. For cellular components a total of 1617 sequences were identified.

Nutrient requirements of paramphistomes have not yet been subject to in-depth study, however the high abundance of FABPs identified in the proteomic profile of many species adult worms are hypothesised to help in the utilisation of the volatile fatty

acids that are created as a by-product from microbial fermentation. This would allow their utilisation as a source of nutrition by the parasites (Belanche *et al.,* 2012; Sripa *et al.,* 2017). As a variety of trematodes are known to not be able to synthesise fatty acid complements, the niche of the rumen is ideal in that the parasite may be able to utilise fatty acids within the rumen and use FABPs in order to transport host fatty acids for lipid oxidation (Ginger & Fairbairn, 1966). This combined with its presence in all lifecycle stages of helminth species such as *O. viverrini* and *S. mansoni* could indicate its vital role in energy production essential for growth, development and reproduction, with fatty acids encompassing all requirements for successful egg production and development (Huang *et al.,* 2012; Sripa *et al.,* 2017). Helminths are unable to synthesis a range of lipids including fatty acids and so these molecules need to be obtained from their respective host environment (McManus & Bryant, 1986), therefore fatty acids binding proteins could be a vital component in infection maintenance by supporting the capture and transport of FABPs (Esteves *et al.,* 1997). As FABPs play a critical role in helminth survival through fatty acid oxidation they could be utilised as potential targets for new anthelmintic drugs and vaccines (Sripa *et al.,* 2017). Its immunogenicity may also allow its use as a mechanism to identify infection with specific parasites (Lee & Yong, 2004; Pankao *et al.,* 2006). FABPs may play a crucial role in anthelmintic activity though movement of the compound to the site in which it can exert its effect successfully (Chuang *et al.,* 2008), with studies showing damage to the parasites tegument following utilisation of FABP derived vaccines leading to effective activation of the host immune response (Figeuroa-Santiago & Espino, 2014; Sirisriro *et al.,* 2002). In contrast, the abundance of FABPs could reduce the effective concentration of anthelmintics through passive binding and localisation of anthelmintics in specific sites (Saghir *et al.,* 2001). FABPs have been identified as a

potential vaccine candidate for schistosomiasis and fascioliasis, with the specific protein of interest Sm-14 currently under pre-clinical trial (Tendler *et al.,* 2015; Ramos *et al.,* 2009). FABPs represent ideal vaccine candidate due to their ability to affect the membrane integrity of the tegument and in turn protein binding therefore impairing the parasites mechanisms of detoxification (Timanova-Atanasova *et al.,* 2004) whilst also reducing fecundity and pathological effects of infections (Hillyer, 2005).

Consequently, the identification of FABPs in the soluble somatic *C. daubneyi* proteome profile allows further research into their potential use in diagnostics and in the development of vaccine candidates.

Previous studies into many helminth species have identified an abundance of cathepsins in their proteomic profiles. Cathepsins have important roles in virulence with major roles in initial entry into the host, migration through host tissue as well as immunomodulatory effects leading to suppression of the host immune response allowing successful establishment (Robinson *et al.,* 2008). Cathepsins in *F. hepatica* and *F. gigantica* have been found to be regulated developmentally allowing successful migration through host tissues as well as involvement in nutrient digestions (Cancela *et al.,* 2008; Meemon *et al.,* 2004; Grote *et al.,* 2018). Interestingly, cathepsins were not identified in either proteomic profiles, absence in these fractions could be due to the final residency of *C. daubneyi* requiring migration through the intestine and not via walls/tissues like other helminth species such as *F. hepatica* and so requiring different proteins in order to successfully migrate through the host as well as differences in feeding requirements leading to their absence/low abundance in these profiles.

Across both the adult and egg proteomes antioxidant proteins were found to be highly represented, allowing speculation that these proteins are not only involved in fluke protection from the immune response, but also in protection of the egg whilst in a free-living environment from oxidative stress, and the xenobiotics they maybe exposed to (Moxon *et al.,* 2010). Proteases are abundant in the adult proteome of *F. hepatica* as has been found in this study (Morphew, 2007; Robinson *et al.,* 2009). However, in-line with previous helminth studies proteases are not abundant in the eggs of *C. daubneyi* (Moxon *et al.,* 2010). Both elucidated profiles revealed hits to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has previously been identified in helminths such as *S. mansoni* and *H. contortus* and is actively involved in the binding to complement-C3 and so providing protection to the worms through inhibition of complement activity (Sahoo *et al.,* 2013; Bergmann *et al.,* 2004). GAPDH has been related to resistance in schistosome infections and so has been highlighted as a potential vaccine candidate (Waine *et al.,* 1993; Wang *et al.,* 2013). Both profiles also revealed the presence of disulphide isomerase, which have been identified in many helminth species which have been found to have a significant role in parasite-host interactions (Cao *et al.,* 2014).

3.5 SUMMARY AND FURTHER RESEARCH

The research carried out in this chapter has successfully resolved both the soluble somatic and egg proteome of adult *C. daubneyi.* Specific protein families of importance have been identified in each of the fractions including FABPs from the soluble somatic and AC/sHSPs from the egg that have the potential to be utilised as biomarkers in diagnosis of infection, as well as possible vaccine candidates and targets for treatment options. Further comparative work with previously resolved flatworm proteomes will facilitate the identification of novel protein families and the multitude of hypothetical and uncharacterised proteins identified can be subject to further investigation, allowing a comprehensive understanding of the fundamental biology of *C. daubneyi* and potential unique mechanisms of establishment.

CHAPTER 4.

CHARACTERISATION OF EXTRACELLULAR VESICLES FROM *CALICOPHORON DAUBNEYI*

4.1 INTRODUCTION

Helminths are a significantly untapped source of biological molecules which have the possibility to be targets for both diagnostics and treatment of infection. Helminths have complex life cycles with their establishment dependent upon manipulation of intra- host environments (Coakley *et al.,* 2016). Extensive research has been carried out on their surface proteins and excretory-secretory (ES) products (Hotez *et al.,* 2008; Toledo *et al.,* 2011), but limited studies have shown a stand-alone parasite derived ES substance active in parasite host interactions, such as Sigma-class GSTs and cathepsin L (Dowling *et al.,* 2010) and so it is likely molecules involved in communication are packaged prior to release (Hewitson *et al.,* 2009). Recent discovery of helminth derived EVs identifies a new avenue of research into parasite establishment and communication and study of these EVs could elucidate key components involved in development of long-term infections and the mechanisms utilised in modulating the immune response.

4.1.1 *Helminth EVs*

Helminths are capable of establishing long-term infections through for example their ability to manipulate the host's immune system leading to favourable conditions for their survival and development, namely though ensuring an anti-inflammatory environment (Maizels *et al.,* 2004; Coakley *et al.,* 2016). Only in recent years has the importance of EVs been noted as carriers of cargo for immuno-modulation, with helminth EVs first reported in the ES products of two trematode species, *Echinostoma caproni* and *Fasciola hepatica* (Marcilla *et al.,* 2012) and the mouse nematode *Heligmosomoides polygyrus* (Buck *et al.,* 2014).

Recent studies have identified the release of EVs by a variety of parasitic organisms, however little is known about the mechanism underpinning EV activity with respect to immuno-modulation or the role their proposed modulation plays in establishing infection (Coakley *et al.,* 2017). Importantly, evidence suggests EVs are the main mechanism of protein exportation in helminths, also containing as cargo mimic host protein pools dependent upon specific parasite species (Marcilla *et al.,* 2012). Thus, with the limited knowledge to date EVs released by several helminth parasites have been found to deliver bioactive molecules to host cells where they modulate host gene expression and supress cytokine formation (Buck *et al.,* 2014), with findings that cargo packaged is developmentally regulated leading to the hypothesis that this regulation allows parasite migration within the definitive host as well as a mechanism of response to the hosts immune response (Cwiklinski *et al.,* 2015).

Many parasitic helminths have previously had their Excretory/Secretory (ES) products extensively studied, however the work on these secretomes was hampered by limited transcriptomic datasets, lack of genome sequencing and lack of intelligence on EVs resulting in previous proteomic investigations representing 'total secretomes' which can now be further divided into ES products and EVs (Cwiklinski *et al.,* 2015). EVs have now been identified in both the insoluble fraction and the tegument of parasites (Marcilla *et al.,* 2012), with several studies showing helminths ability to modulate host innate immune cells through successful transfer of bioactive molecules to their targets (Buck *et al.,* 2014; Wang *et al.,* 2015). Further to their role in modulation, parasite derived EVs have been shown to be a key component in the establishment and maintenance of infections, with nematode EVs shown to supress the TH2 immune

response as well as containing miRNAs capable of supressing cytokine production (Cwiklinski *et al.*, 2015; Buck *et al.*, 2014).

Investigations into EVs are now widely carried out due to their recognised role in inter-cellular communication through their ability to transfer proteins, lipids, mRNA, microRNA and non-coding RNA to their targets (**Figure 4.1**) (Andaloussi *et al*., 2013; Record., 2014). However, a key limitation in proteomic studies of helminths (and their EVs) is the lack of genomic data available, therefore transcriptomic data sets and subsequent BLAST analysis need to be utilised in order to identify protein function (Garg *et al.*, 2013). Recent developments in omic techniques have allowed identification of immunomodulatory proteins packaged inside EVs, however further investigation into EV production and interaction could identify new methods of parasite control (Torre-Escudero & Robinson, 2017), with these developments in proteomics investigations already allowing identification of potential vaccine candidates (Sotillo *et al.*, 2016).

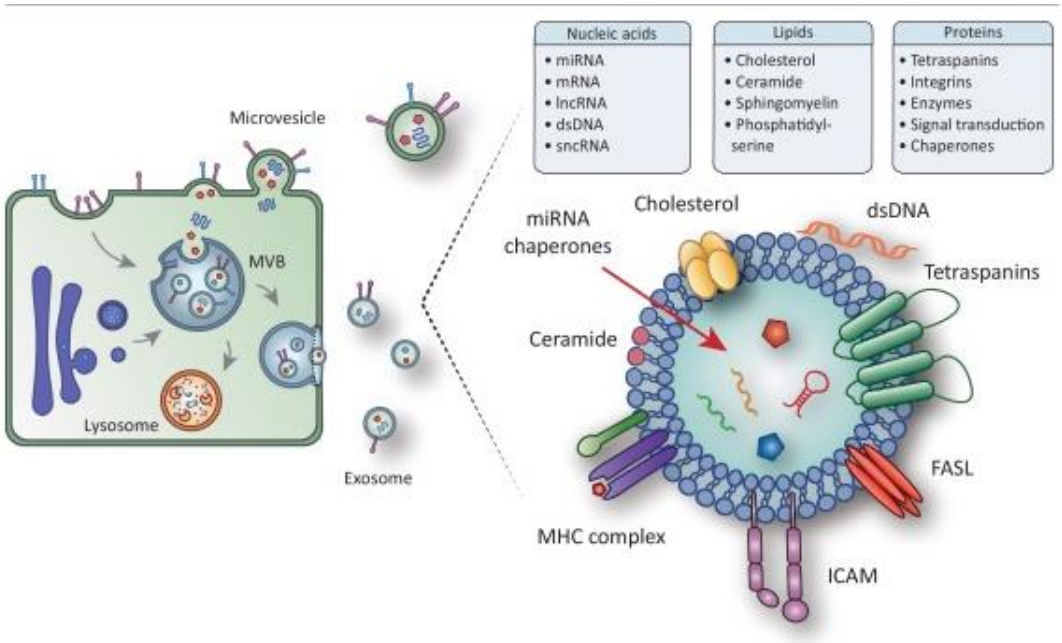


Figure 4.1*:* Extracellular vesicle composition. Generic layout of an extracellular vesicle and their membrane components including internalised compounds (Veerman *et al.*, 2019).

4.1.2 *EV Proteomics*

Proteomic investigation into trematode EVs remains problematic due to the absence of sequenced genomes, however, transcriptomic datasets can be utilised to interpret results. Transcriptomics combined with advances in quantitative proteomics has allowed elucidation of potential biomarkers and their subsequent validation as vaccine candidates (Fujita *et al.,* 2017). Combined with developments in LC-MS/MS and incorporation of iTRAQ technology, high-throughput proteomics can now be implemented in the proteomic study of EVs.

Both packaged protein cargo and membrane associated proteins have been utilized as markers allowing positive characterisation of EVs populations. These proteins also allow identification of cellular location, origin and mechanism of biogenesis, with dedicated online databases cataloguing these components, such as ExoCarta (Simpson *et al.,* 2012). Generally, EVs have been found to be highly abundant in cytoskeletal, cytosolic, membrane and heat shock proteins, however proteomic profiles can be variable dependent upon the mechanism of vesicles isolation as well as method of characterisation making it difficult to carry out comparative work between studies (Yanez-Mo *et al.,* 2015; Mekonnen *et al.,* 2018).

Subgroups of EVs may contain different contents dependent upon stimulation of biogenesis as well as the composition of multivesicular bodies (MVBs) within releasing cells resulting in differences in contents packaged (Aatonen *et al.,* 2014), with differences in composition *in vitro* dependent upon the conditions in which parasites are cultured (Kucharzewska & Belting, 2013). Due to differences in composition between subpopulations, there is no single marker that can be utilized in order to universally characterise EVs; reasons for the difference in protein

composition remain unknown (Yanez-Mo *et al.*, 2015; Matheiu *et al.*, 2019). These unknowns mean there is currently no mechanism of purifying a guaranteed pure sub-population and so caution needs to be taken when analysing EVs proteomic data (Veerman *et al.*, 2019).

Mass spectrometry of EVs has drastically improved understanding of their cargo by elucidating both mechanisms of biogenesis and physiological function (Choi *et al.*, 2013). Early studies into EVs utilised separation through gel electrophoresis followed by targeted MS, however now high throughput LC-MS/MS is carried out combined with initial separation of EVs from samples. Developments in proteomics has found that proteins packaged within EVs are not linked to the abundance of proteins within the releasing cell but instead controlled through protein sorting mechanisms during EV biogenesis (Choi *et al.*, 2013).

4.1.3 *Antimicrobial effects of EVs*

Helminth parasites have long been known to have immunomodulatory effects on their hosts through their ability to excrete a range of bioactive molecules known to exert their immunomodulatory effect on defined targets (Eberle *et al.*, 2015). Helminths share a niche with an array of bacterial species due to their localisation in the gastrointestinal tract, and so modify the microbiota of the host leading to modification of the host immune response (Brosschot & Reynolds, 2018). Early studies into membrane vesicles (MVs) from bacteria showed their potential antimicrobial activity with studies identifying their ability to deliver toxins to recipient cells (Mashburn-Warren & Whiteley, 2006; Li *et al.*, 1996).

Recent studies of model helminth species which also inhabit a microbial environment have identified the presence of antimicrobial peptides (AMPs) in ES products hypothesised to be released in order to allow development and regulation of a conditionally desirable microbiota (Midha *et al.,* 2018). As well as identification of AMPs in ES products, treatment of the helminth parasite *A. suum* with a range of both gram-positive and gram-negative bacteria was found to induced transcription of AMPs, suggesting that the synthesis of defence molecules could be constitutive (Pillai *et al.,* 2005). Studies have also identified the presence of AMPs in EVs, including several parasites actively stimulating AMP containing EVs from their hosts (Hu *et al.,* 2013; Wang *et al.,* 2015). Fungal released EVs have also been investigated and found to induce antimicrobial effects in recipient host cells though stimulation of antimicrobial compound synthesis (Oliveira *et al.,* 2010).

Exosomes have been acknowledged as innate immune effectors with roles in defence. However, their effects are dependent upon structural integrity and the conditions in which biogenesis takes place (Hiemstra *et al.,* 2014). Studies have identified the antimicrobial peptides in exosomes released from certain cell types, with their interactions leading to restriction of bacterial growth (Timar *et al.,* 2012). As well as the AMP released by helminth parasites, host proteins secreted by the gut have also been shown to alter the gut microbiota through delivery of AMPs (Hu *et al.,* 2013; Smythies & Smythies, 2014). There have been numerous studies into the adaptation of the immune response by helminth ES products, however no studies to date have specifically targeted EVs in the study of immuno-regulation and their effect on microbial populations. Research into the interaction of helminth species and their effect on the host microbiota remain vastly unexplored (Midha *et al.,* 2018).

Although it is well understood that helminths have immunomodulatory activity, it is still unknown if this mechanism of evasion requires interaction with the microbiota and if the alteration of physiology, permeability and secretions in helminths has a direct effect on microbial species present and their spatial organisation (Zaiss & Harris, 2016). Currently, there are many studies into the effect of ES products on the gut microbiota and subsequent evasion of immune response, however there are no studies explicitly identifying EVs as a method through which the microbiota is regulated. We hypothesise that rumen fluke EVs contain antimicrobial peptides that contribute to modification of rumen microbiota, allowing the establishment of infection.

4.1.4 *CHAPTER AIMS*

This experimental chapter is the first in-depth proteomic and transcriptomic study into *C. daubneyi* extracellular vesicles and their potential effects on bacterial populations.

Therefore, the aims of this chapter are:

- Identification of extracellular vesicles in *C. daubneyi* excretory-secretory (ES) products.
- Proteomic characterisation of *C. daubneyi* EVs released during *in vitro* culture and subsequent assignment of resolved proteins functionality.
- Proteomic characterisation of membrane associate EV proteins.
- Analysis of EVs antimicrobial properties and their effect on microbial populations.
- Bioinformatic investigation of *C. daubneyi* transcriptome allowing identification of EV biogenesis proteins and elucidation of the mechanisms behind EV formation and release.

4.2 METHODS

Unless stated otherwise, all methods were carried out as stated in **Chapter 2**. All solutions were made up using ddH_2O and molecular grade reagents.

4.2.1 *Size exclusion chromatography (SEC) purification*

As described by Davis *et al.* (2019), EVs with maintained functionality were purified utilising size exclusion chromatography (SEC). Following parasite maintenance, DMEM media was centrifuged at 300 × *g* for 10 minutes at 4°C, supernatants were removed and centrifuged at 700 × *g* for 30 minutes at 4°C removing large debris. The resulting media was concentrated in 30 kDa MCWO Amicon Ultra-15 centrifuge filters (Millipore, U.K.) through centrifugation at 4000 × *g* for 20 minutes at 4°C until ~500 µl of sample remained. Concentrated samples were passed over a qEV Size Exclusion Chromatography column (IZON) according to the manufacturer's instructions. Briefly, columns were equilibrated with 10 ml of 0.2 µm syringe filtered PBS. Sample was then passed over the column with the addition of PBS following filter penetration. The first 2.5 ml was stored as flow through and the following 1.5 ml was collected as the EV sample. Sample was stored at -80°C for downstream analysis.

4.2.2 *Particle size/concentration quantification*

Isolated EVs were placed in a nanopore (NP200, Izon Science) within a qNano particle analyser (IZON Science). Samples were analysed following calibration and measured at 47 mm nanopore stretch at 100 nA under 7 mbar pressure. Particles were detected through short pulses of current before subsequent analysis with qNano particulate analysis software (Izon, version 3.2).

4.2.3 *Transmission Electron Microscopy (TEM) – Grid preparation and imaging*

EVs were fixed to formvar/carbon coated copper grids (agar scientific) through addition of 10 µl of sample to each grid on ice for 45 minutes. Grids were then transferred onto the meniscus of 4% w/v uranyl acetate for 5 minutes. Grids were transferred into a storage container for at least 24 hours before imaging and stored at room temperature. Analysis of previously prepared grids was carried out on a Joel 1010 transmission electron microscope at 80 kV. Images were taken digitally, and further size analysis used to distinguish extracellular vesicles within the samples (30-100 µm).

4.2.4 *Trypsin Shave of EVs*

SEC purified EVs were first aliquoted into their respective volumes containing 200 µg of protein, and either diluted to 250 µl total volume or placed in a 30 kDa MWCO Amicon filter and centrifuged following the manufacturers guidelines until only 250 µl remained. In brief, sample was added to the filter and placed within a collection tube, samples were centrifuged at 14, 000 × *g* for 10 minutes at 4°C. This was repeated until 250 µl remained. Samples were aspirated before removal into an Eppendorf. Trypsin (sequencing grade modified trypsin, Roche, U.K) was dilute to 100 µg/ml and added to the EVs at a final concentration of 50 µg/ml. Samples were incubated for 5 minutes at 37°C followed by centrifugation for 1 hour at 100,000 × *g* at 4°C (S55-S rotor, Sorval MX120 centrifuge, Thermo Scientific). The resulting supernatant was aliquot into 20 µl fractions and stored at -20°C prior to gel free mass spectrometry analysis. Samples were injected at 1 µl per sample and analysed accordingly (**Section 2.12).**

4.2.6 Microbial culture

In total 10 microbial species were tested for the effects of *C. daubneyi* EVs on them (**Table 4.1**). In brief, each species was grown in broth overnight and 1 ml of each aliquot and added to 50 µg/ml of EVs. Interaction was allowed overnight before subsequent plating. Each species was also subject to a control with no addition of EVs. Each control and strain were then dilute 1:1000, 1:10000 and 1:100000 and plated to grow over night before subsequent colony counting after 24 hours.

Table 4.1: Microbial organisms used in this study.

Species	Strain	Order	Identifier
Escherichia coli	Negative	Enterobacteriales	ATCC 25922
Klebsiella pneumoniae	Negative	Enterobacteriales	ATCC 700603
Proteus mirabilis	Negative	Enterobacteriales	NCTC 10975
Pseudomonas aeruginosa	Negative	Pseudomonadales	ATCC 27853
Staphylococcus aureus	Positive	Bacillales	ATCC 29213
Staphylococcus epidermidis	Positive	Bacillales	NCTC 11047
Staphylococcus saprophyticus	Positive	Bacillales	Wild strain
Enterococcus faecalis	Positive	Lactobacillales	ATCC 29212
Bacillus subtilis	Positive	Bacillales	ATCC 6633
Candida albicans	Positive	Saccharomycetales	NCTC 3255

4.2.5 *Bioinformatic interrogation of biogenesis proteins – sequence retrieval pipeline*

In brief, literature was searched in order to identify proteins involved in EV biogenesis in both closely related helminths and humans through searching of the GenBank database (http://www.ncbi.nlm.nih.gov/). In this experiment sequences involved in the ESCRT-dependent and ESCRT-independent pathway were identified as well as proteins involved in subsequent abscission. In total a database of 89 proteins was assembled and submitted to local blast analysis following the method of (Altschul *et al.*, 1990) in order to identify putative homologs of biogenesis proteins in the *C. daubneyi* transcriptome. BLAST searches were all carried out using BioEdit (Hall, 1999) with a cut off value of E^{-20} set for each search and only results over this value included as positive identification.

4.3 RESULTS

4.3.1 *TEM identification of EVs in C. daubneyi ES products*

Presence of extracellular vesicles in both differential centrifugation (DC) and SEC isolated samples was confirmed by identification of membrane bound vesicle ~30-100 nm in size through transmission electron microscopy (TEM) (Nowacki *et al.,* 2015). Random sample selection from each collection was utilised to ensure EV presence across samples. TEM imaging identified that the EVs present had diverse morphologies with ruptured vesicles only identified in DC purified samples. A large number of aggregated vesicles were also identified in the DC samples whilst no aggregation was observed in the samples isolated through SEC (**Figure 4.2**). Despite 0.2 µm filtering background contamination of non-EV products was still present. Comparison with published literature allowed morphological identification of a mix of both microvesicle and exosome subpopulations.

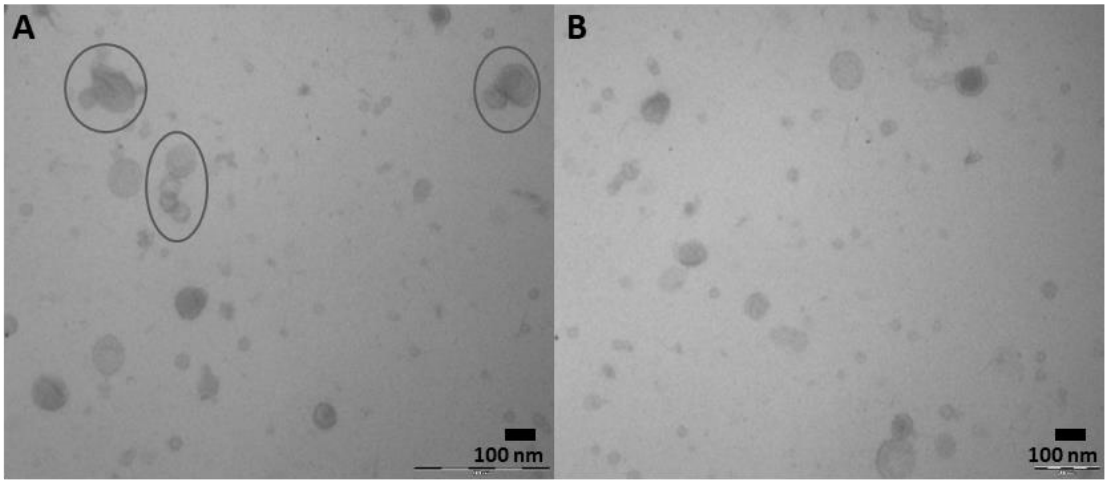


Figure 4.2: Developed TEM micrographs identifying extracellular vesicles secreted by *C. daubneyi* through DC and SEC isolation. (A) DC purified samples showing aggregation of vesicles (outlined) (B) SEC purified samples showing no aggregation.

4.3.2 *Visualisation of C. daubenyi EV proteome*

Following positive identification of DC EVs, samples were lysed to allow quantification and a concentration gradient of 5, 10, 15, 20 and 25 µg was run on a 12.5% polyacrylamide gel determining the optimum concentration for resolution of EVs proteins. 25 µg of EV protein was determined as the optimum quantity with optimal visibility following staining and so this was carried forward for further protein analysis. In total 3 samples of *C. daubneyi* EVs (n=3) were run out on a 12.5%, 1-Dimensional polyacrylamide gel (**Figure 4.3**). A low molecular weight marker (Biorad, UK) was used in order to deduce the weights of the protein bands resolved and the resulting gel was stained using coomassie brilliant blue.

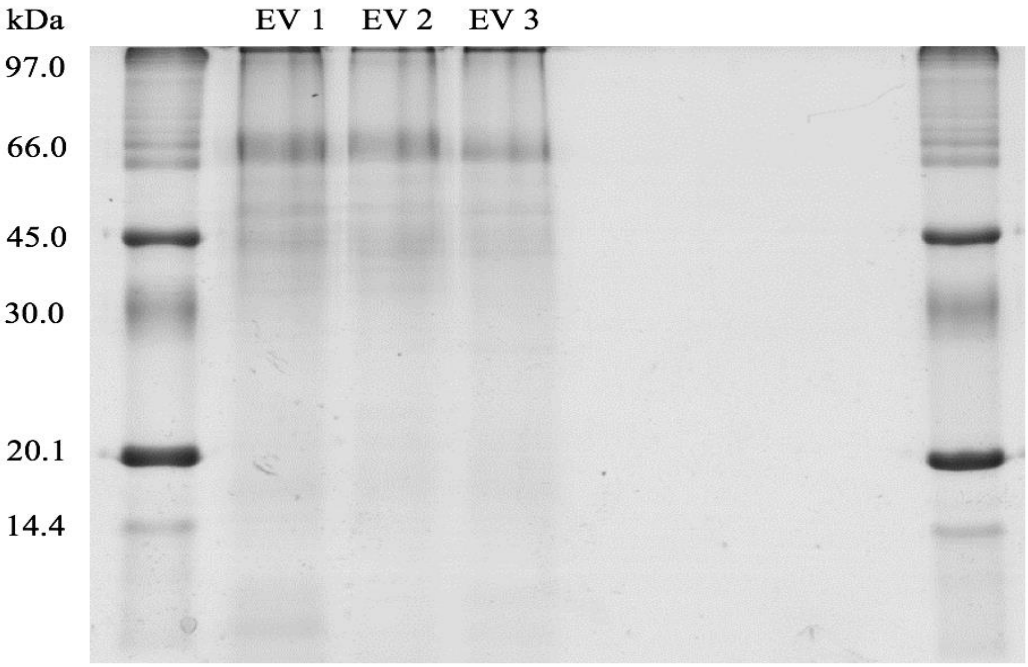


Figure 4.3: 1D 12.5% polyacrylamide gel of *C. daubneyi* EV protein (n=3).

4.3.3 *Mass spectrometry of C. daubneyi EVs*

Each of the 3 replicate lanes shown in **Figure 4.3** were divided into 11 bands and subject to trypsin digestion prior to LC-MS/MS analysis. Following MS analysis results from each of the 11 samples for each lane for each replicate were combined to elucidate the complete EV proteome. Transcript hits identified in each replicate were sorted and only those with a score over the significance threshold of 49 were included as positive hits. In total 378 proteins consistent across all three replicates were identified. Each of these transcript identifiers were searched in the transcriptome and their sequences translated (http://expasy.com) in order to find the relevant open reading frame (ORF). Protein sequences were then submitted to BLASTp analysis (https://www.ncbi.nlm.nih.gov) allowing identification of homologous sequences in the NCBI database. The protein ID, species ID and accession number for each of these hits were recorded. All results were quantified by the number of unique peptides returned from LC-MS/MS and sorted to elucidate the top 100 protein hits **Table 4.2.**

The most abundant proteins identified in the EV proteome were ATPase family protein (TR26097|c0_g1), beta tubulin (TR18968|c0_g1 and TR17099|c2_g1) and unknown protein - TR21569|c0_g5. A selection of common EV markers were also identified within the top 100 proteins through comparison with the ExoCarta database (http://exocarta.org), including glyceraldehyde-3-phosphate dehydrogenase, actin, 14-3-3, annexin A7 and alpha/beta tubulins confirming characterisation as EVs. Several proteins were identified numerous times within biological replicates, these repeated hits are likely due to post-translational modifications (PMTs) which are common in eukaryotes (Beltrao *et al.*, 2013). Due to the absence of a genome, the top BLAST hits species were investigated. Species results for all proteins resolved were then collated in order to allow visualisation number of hits to each species.

Unsurprisingly, 8 out of the top 10 species to which proteins were assigned were all closely related trematode species (**Figure 4.4**) including *Opisthorchis viverrini, Clonorchis sinensis, Fasciola hepatica* and several *Schistosoma spp.* accounting for 79.8% of the proteins identified. Although 79.8% of hits were assigned to 8 closely related trematode species, the remaining 20.2% encompassed a further 50 non-trematode species.

Table 4.2: Top 100 proteins resolved in *C. daubneyi* extracellular vesicles following blast analysis of transcript identifiers. Proteins were ranked based on the number of unique peptides hit during tandem MSMS and blast identifiers chosen based off on E-value.

Transcript ID	Isoform	Unique peptides	Blast Description	Organism	NCBI Accession
TR26097|c0_g1	i1	76	ATPase family protein	*Opisthorchis viverrini*	OON14744.1
TR18968|c0_g1	i1	58	Tubulin beta-3	*Fasciola hepatica*	CAP72051.1
TR17099|c2_g1	i1	57	Tubulin beta	*Clonorchis sinensis*	GAA51682.1
TR21569|c0_g5	i1	44	-	-	-
TR9358|c0_g1	i1	39	Actin	*Gossypium arboreum*	XP_017626052.1
TR19715|c0_g1	i1	36	Radixin	*Carlito syrichta*	XP_008054748.1
TR21569|c0_g5	i2	36	-	-	-
TR17877|c2_g2	i1	31	Alpha-tubulin	*Fasciola hepatica*	CAO79602.1
TR18958|c0_g1	i1	28	Alpha tubulin	*Schistosoma japonicum*	AAW27478.1
TR19159|c0_g1	i1	26	Alpha tubulin	*Clonorchis sinensis*	GAA56421.1
TR23254|c0_g1	i1	24	Leucyl aminopeptidase	*Clonorchis sinensis*	ABL11479.1
TR24554|c0_g1	i1	23	alpha-glucosidase	*Schistosoma mansoni*	XP_018647945.1
TR18070|c0_g1	i1	22	Acid sphingomyelinase phosphodiesterase	*Clonorchis sinensis*	GAA33847.2
TR23757|c0_g1	i1	22	Alpha tubulin	*Clonorchis sinensis*	GAA38337.2
TR24153|c0_g1	i1	22	Hypothetical protein	*Opisthorchis viverrini*	OON14506.1
TR23969|c0_g1	i1	21	Tektin	*Clonorchis sinensis*	GAA33438.1
TR23279|c0_g1	i1	21	Alpha tubulin	*Fasciola hepatica*	CAO79606.1
TR18525|c0_g1	i1	20	14-3-3 epsilon	*Opisthorchis viverrini*	OON22058.1
TR21014|c0_g1	i1	20	SNaK1	*Schistosoma mansoni*	AAL09322.1
TR20466|c0_g1	i1	19	Calpain	*Schistosoma mansoni*	CCD74981.1
TR20643|c0_g1	i1	18	annexin a7	*Schistosoma haematobium*	KGB33756.1
TR18939|c0_g1	i1	18	-	-	-
TR22034|c1_g4	i3	18	Aldolase	*Opisthorchis viverrini*	OON20700.1
TR25036|c3_g1	i13	17	Cathepsin D	*Fasciola gigantica*	AEE69372.1
TR25036|c3_g1	i2	17	Cathepsin D	*Fasciola gigantica*	AEE69372.1
TR23288|c0_g1	i1	16	EF-hand domain	*Schistosoma mansoni*	CCD76447.1
TR19073|c1_g2	i1	16	Hypothetical protein	*Opisthorchis viverrini*	OON16570.1

Table 4.2: *continued.*

TR18454lc0_g1	i1	16	Hypothetical protein	*Opisthorchis viverrini*	OON20759.1
TR25036lc3_g1	i1	16	eukaryotic aspartyl protease	*Opisthorchis viverrini*	OON23093.1
TR21569lc0_g5	i7	16	-	-	-
TR25036lc3_g1	i14	14	Cathepsin D	*Clonorchis sinensis*	GAA56870.1
TR23782lc0_g2	i1	14	Leukotriene-A4 hydrolase	*Clonorchis sinensis*	GAA49617.1
TR17046lc0_g1	i1	15	14-3-3 epsilon	*Clonorchis sinensis*	AEO89649.1
TR9216lc0_g1	i1	15	14-3-3 protein	*Opisthorchis viverrini*	OON14987.1
TR25395lc0_g2	i2	15	Hypothetical protein	*Opisthorchis viverrini*	XP_009165006.1
TR17779lc0_g1	i1	15	Actin	*Opisthorchis viverrini*	XP_009173847.1
TR25395lc0_g1	i1	15	Hypothetical protein	*Opisthorchis viverrini*	XP_009165006.1
TR22003lc1_g5	i1	14	Tubulin beta	*Cricetulus griseus*	XP_007606483.1
TR19892lc0_g1	i1	14	Hypothetical protein	*Opisthorchis viverrini*	OON16605.1
TR18374lc0_g1	i1	14	Triose phosphate isomerase	*Fasciola hepatica*	AGJ83762.1
TR25036lc3_g1	i12	14	Cathepsin E-A	*Apaloderma vittatum*	KFP91951.1
TR17173lc0_g1	i1	14	leishmanolysin peptidase	*Clonorchis sinensis*	GAA54636.1
TR17164lc0_g1	i1	13	glyceraldehyde 3- phosphate	*Clonorchis sinensis*	GAA28380.1
TR15297lc0_g1	i1	13	Chloride intracellular channel	*Clonorchis sinensis*	GAA38512.2
TR19675lc0_g1	i1	13	JF-2	*Schistosoma japonicum*	AAB49033.1
TR25036lc3_g1	i8	13	Cathepsin D	*Clonorchis sinensis*	GAA56870.1
TR23072lc0_g1	i1	13	EF-hand calcium-binding domain	*Clonorchis sinensis*	GAA51832.1
TR24199lc0_g4	i1	12	Plastin-1	*Clonorchis sinensis*	GAA29911.1
TR21252lc0_g1	i1	12	33kDa inner dynein arm light chain	*Schistosoma japonicum*	CAX73643.1
TR25837lc0_g3	i3	12	EF-hand domain-containing family	*Clonorchis sinensis*	GAA35263.2
TR24356lc0_g1	i1	12	Programmed cell death 6	*Clonorchis sinensis*	GAA36539.2
TR16926lc0_g1	i1	12	Hypothetical protein	*Opisthorchis viverrini*	XP_009176015.1
TR19294lc0_g1	i1	12	14-3-3 epsilon	*Bemisia tabaci*	XP_018901647.1
TR21578lc0_g1	i1	11	BTB/POZ domain protein	*Clonorchis sinensis*	GAA34922.2
TR22249lc0_g1	i1	11	Beta tubulin	*Clonorchis sinensis*	GAA34286.2

Table 4.2: *continued.*

TR24268|c0_g1	i1	11	Enolase	*Clonorchis sinensis*	AAN03783.1
TR25036|c0_g1	i1	11	Lysosomal aspartic protease	*Trachymyrmex*	XP_018348387.1
TR21435|c0_g1	i1	11	Hypothetical protein	*Clonorchis sinensis*	GAA51305.1
TR12225|c0_g1	i1	10	SPFH Domain	*Schistosoma mansoni*	XP_018653106.1
TR16536|c0_g1	i1	10	14-3-3 protein beta/alpha	*Schistosoma haematobium*	XP_012796666.1
TR22215|c0_g1	i1	10	Cytosolic dipeptidase	*Clonorchis sinensis*	GAA53702.1
TR25911|c3_g3	i1	10	Alpha glucosidase	*Schistosoma mansoni*	XP_018647945.1
TR16097|c0_g1	i1	10	Chloride intracellular channel	*Clonorchis sinensis*	GAA38512.2
TR16484|c0_g1	il	10	tyrosine 3-monooxygenase/tryptophan	*Clonorchis sinensis*	GAA36880.2
TR26107|c1_g1	i1	10	Dyenin heavy chain	*Clonorchis sinensis*	GAA49374.1
TR22250|c0_g2	i2	10	Multidrug resistance protein	*Schistosoma haematobium*	XP_012800768.1
TR22248|c1_g1	i1	10	Alpha tubulin	*Clonorchis sinensis*	GAA29180.2
TR17640|c0_g1	i1	10	-	-	-
TR23598|c0_g1	i1	9	Hypothetical protein	*Opisthorchis viverrini*	XP_009164230.1
TR23598|c0_g1	i2	9	Hypothetical protein	*Opisthorchis viverrini*	XP_009164230.1
TR24469|c1_g4	i3	9	LAMA-like protein precursor	*Schistosoma japonicum*	CAX74467.1
TR20794|c0_g1	i1	9	Phosphoglycerate kinase	*Fasciola hepatica*	AAZ17561.2
TR25036|c3_g1	i11	9	Cathepsin D	*Fasciola gigantica*	AEE69372.1
TR20146|c0_g1	i2	9	-	-	-
TR21218|c0_g5	i2	9	Alpha tubulin	*Clonorchis sinensis*	GAA40522.2
TR25036|c3_g1	i3	9	Lysosomal aspartic protease	*Bactrocera cucurbitae*	XP_011196136.1
TR22034|c0_g1	i1	9	Fructose-bisphosphate aldolase	*Clonorchis sinensis*	GAA28648.2
TR25911|c3_g1	i1	8	Alpha glucosidase	*Schistosoma mansoni*	XP_018647945.1
TR24356|c0_g1	i1	8	Programmed cell death 6-interacting	*Clonorchis sinensis*	GAA36539.2
TR21664|c1_g2	i10	8	Alpha tubulin	*Oreochromis niloticus*	XP_019205265.1
TR21218|c0_g5	i2	8	Alpha tubulin	*Clonorchis sinensis*	GAA40522.2
TR21854|c0_g1	i1	8	V-type H+- transporting ATPase subunit	*Clonorchis sinensis*	GAA37292.2
TR23054|c0_g1	i1	8	Tektin	*Clonorchis sinensis*	GAA54519.1

Table 4.2: *continued.*

TR24109|c0_g1	i1	8	Coiled-coil domain-containing protein	*Schistosoma haematobium*	XP_012800027.1
TR22803|c1_g1	i2	8	annexin a11	*Clonorchis sinensis*	GAA33818.2
TR14290|c0_g1	i1	8	Xaa-Pro dipeptidase	*Clonorchis sinensis*	GAA56661.1
TR22248|c0_g1	i1	8	Alpha tubulin	*Kryptolebias marmoratus*	XP_017293878.1
TR25150|c0_g1	i1	8	Arrestin 1	*Echinococcus granulosus*	EUB60491.1
TR20600|c1_g1	i1	8	Beta arrestin	*Clonorchis sinensis*	GAA50412.1
TR15860|c0_g1	i1	8	Yolk ferritin	*Paragonimus westermani*	AAK35224.1
TR20126|c1_g1	i3	8	Alpha tubulin	*Brassicogethes aeneus*	AGQ51765.1
TR17297|c0_g1	i1	9	Hypothetical protein	*Opisthorchis viverrini*	OON19254.1
TR26054|c0_g1	i1	9	Dyenin beta chain	*Clonorchis sinensis*	GAA54008.1
TR23304|c0_g2	i1	9	WD repeat-containing protein	*Clonorchis sinensis*	GAA56527.1
TR21815|c0_g1	i1	7	Hypothetical protein	*Opisthorchis viverrini*	XP_009176424.1
TR19914|c0_g1	i1	7	Hypothetical protein	*Opisthorchis viverrini*	XP_009174760.1
TR14088|c0_g1	i1	6	Malate dehydrogenase	*Clonorchis sinensis*	GAA34201.2
TR16082|c0_g1	i1	6	Glycerol kinase	*Opisthorchis viverrini*	OON13409.1
TR18383|c0_g1	i1	6	SJCHGC04509 protein	*Schistosoma japonicum*	AAX25214.2
TR17290|c0_g1	i1	4	Hypothetical protein	*Hymenolepis microstoma*	CDS26448.1

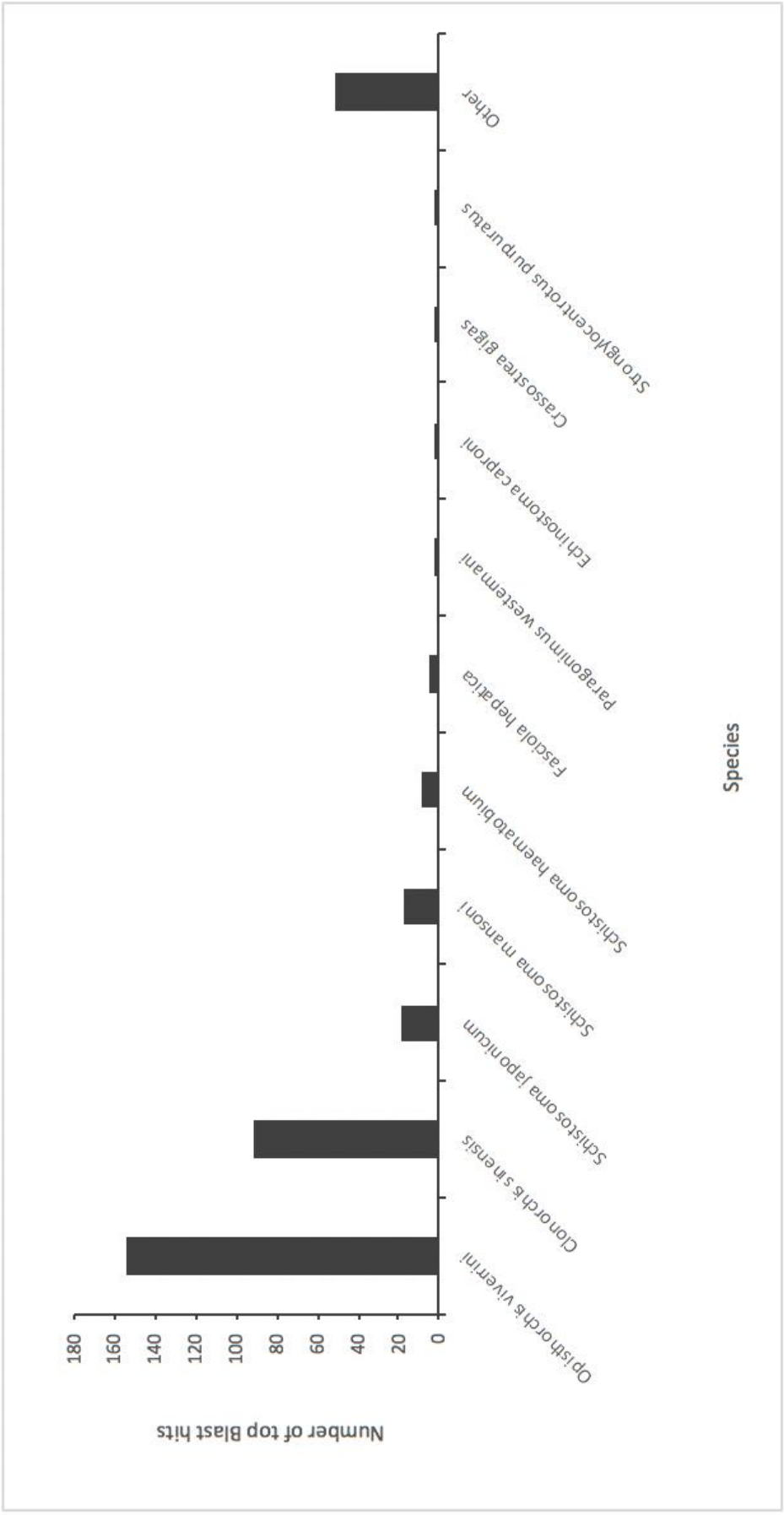


Figure 4.4: Top 10 species identified in Blast analysis of *C. daubneyi* EVs and the number of the proteins to which they were assigned. 'Other' includes all other species that were identified outside of the top 10.

4.3.4 *Characterisation of C. daubenyi EV proteome*

All 378 sequences resolved in the EV proteome were further characterised by their functionality through use of the Pfam and Interpro databases and sorted into 9 distinct categories: Cytoskeleton, Proteases, Enzymes, Chaperones, Metabolism, Transporters, Carrier, Exosome Biogenesis and Others as previously described by (Cwiklinski *et al.,* 2015) (**Figure 4.5)**. Interestingly the category with the greatest number of sequences assigned was 'other' encompassing all sequences with no blast result or a blast result to a hypothetical or unassigned protein accounting for 34% of the sequences, followed by cytoskeletal proteins accounting for 24% of proteins. The category representing the fewest number of proteins was carriers only accounting for 1% of the proteome.

Each protein sequence identified in the whole proteome of DC EVs were then submitted to GeneOntology analysis (http://geneontology.com), identifying a large number of biological process, molecular function and cellular component categories to which the proteins could be assigned, the top 20 of which are detailed in **Figure 4.7**. For Molecular function the most frequent category to which proteins were assigned was ion binding, for Biological process they were primary metabolic process and organic substrate biological process and for cellular components intercellular had the top number of proteins. Biological processes returned the greatest number of categories, whilst cellular components had the least, with sequences categorised into more than one functional category. Unsurprisingly GO categories for extracellular space, plasma membrane, organelle membrane and membrane protein complex were within the top 20 results and are the main functional categories to which EV proteins are assigned.

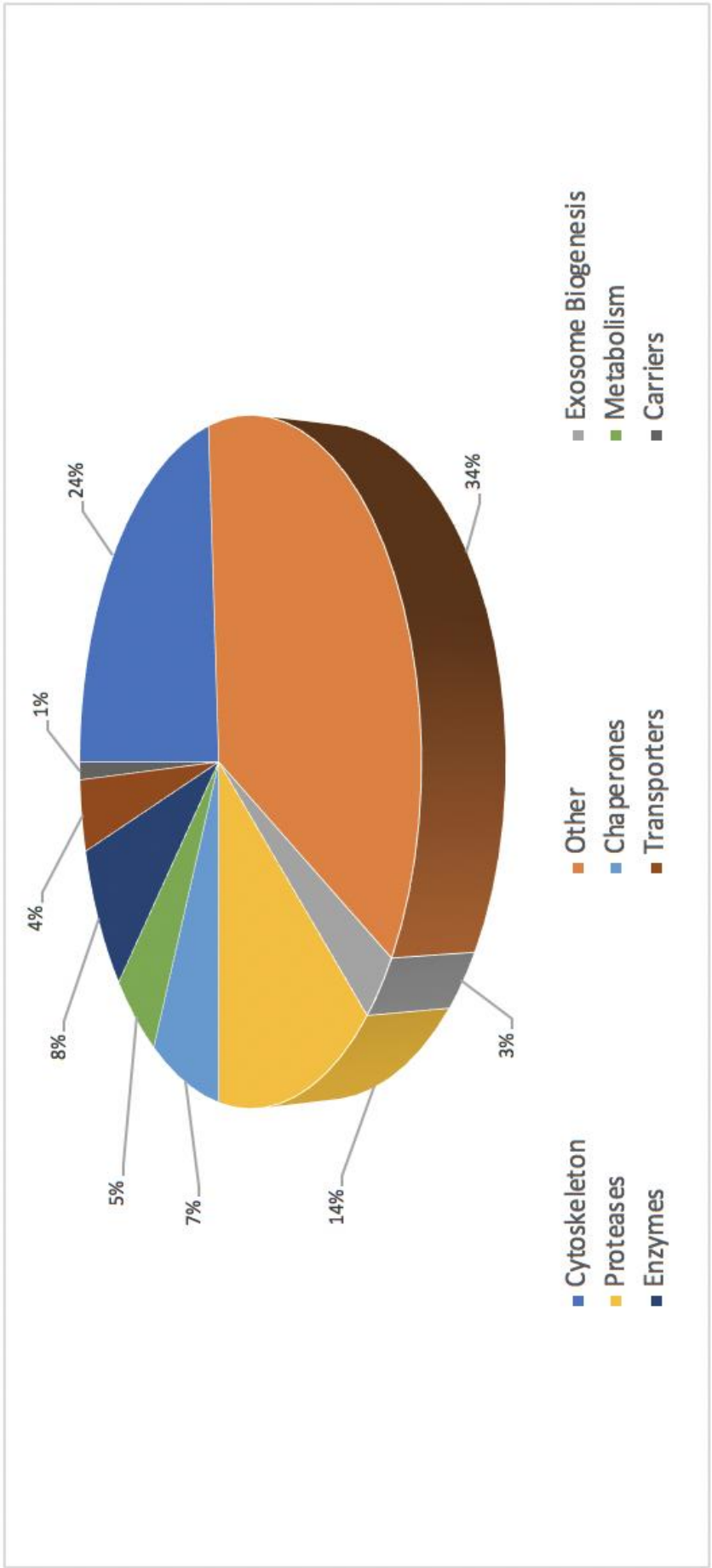
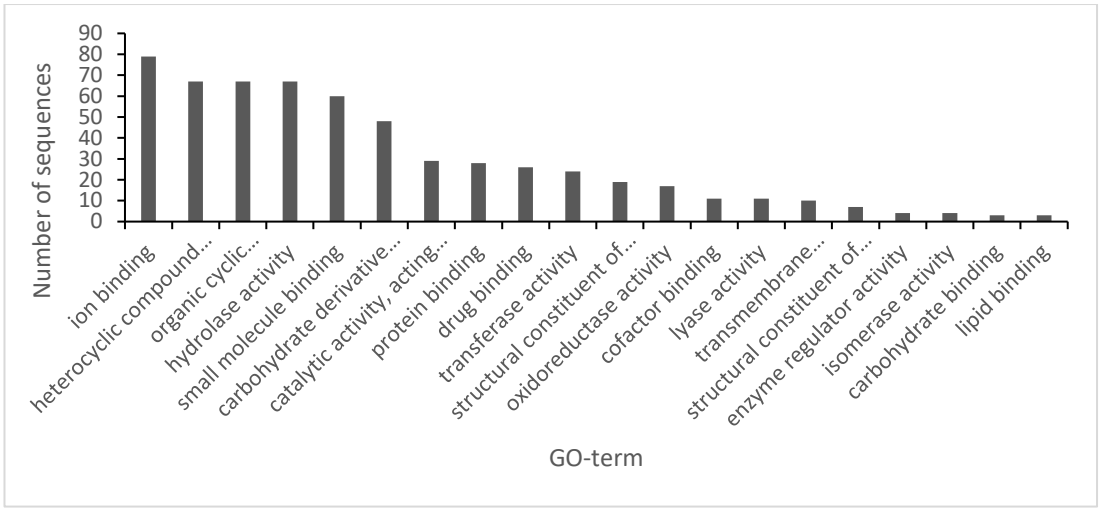
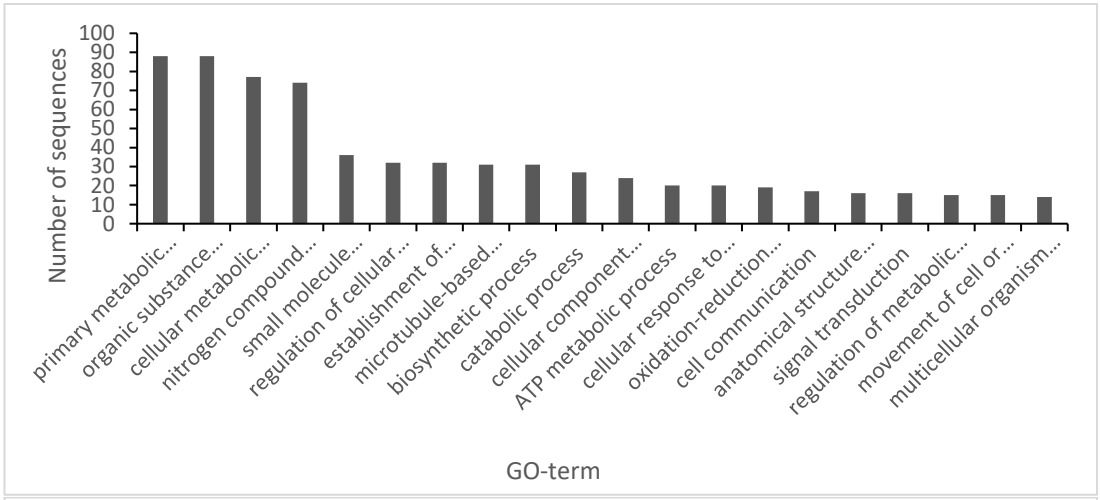


Figure 4.5: Categorisation of all sequences returned from the EV proteome. Proteins consistent across three replicates were submitted to Pfam and GeneOntology searches and assigned to 9 functional categories as defined by Cwiklinski *et al.*, 2015. Proteins that did not fit any of the nine categories were placed into a 10th category named 'other'. Cytoskeleton associated proteins accounted for 24% of the sequences resolved, Proteases 14%, Enzymes 8%, Chaperones 7%, Transporters 4%, Exosome biogenesis 3%, Metabolism 5%, Carriers 1% and Others 34%.





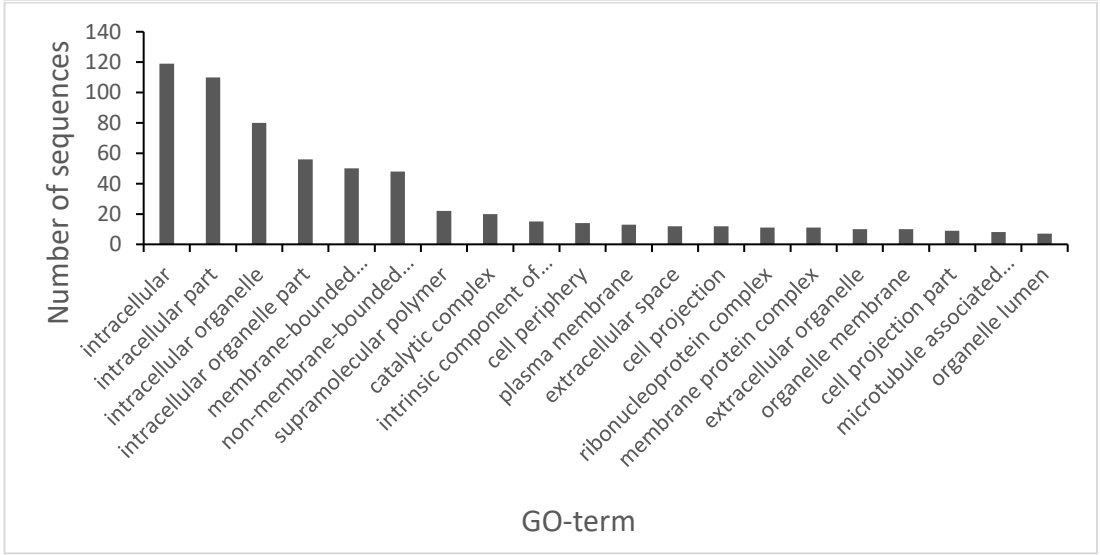


Figure 4.7: Top 20 level 3 gene ontology descriptions of *C. daubneyi* EV proteins isolated through SEC. Classified by Molecular function (A) Biological processes (B) and Cellular component (C) functional categories.

4.3.5 *Surface proteins of C. daubneyi EVs*

Following resolution of an EV proteomic profile, the proteins present on the external surface of EVs were investigated through trypsin cleavage from the membrane. Mass spectrometry run time was also investigated in order to optimise the run length required to identify the greatest number of proteins when utilising a gel free MS approach, the results of the trypsin shave and run time optimisation are outlined in **Table 4.3**. Each sample was submitted to a 5-minute trypsin shave and run on the mass spectrometer for 30, 45 and 70 minutes in order to optimise run time for optimum protein hits returned. 70 minutes was shown to include all of the hits from the 30- and 45-minute run and so is the dataset carried forward for analysis. Transcript IDs identified through MSMS were translated before being submitted to BlastP investigation allowing identification of the protein IDs.

4.3.6 *Antimicrobial potential of C. daubneyi EVs*

Following identification of surface proteins, EVs isolated through SEC were utilised in investigating their effect on microbial populations. 10 microbial organisms (**Table 4.1**) were treated with 250 μg of EVs before serial dilution of 1:1000, 1:10000 and 1:100000 and allowed to incubate for 1 hour before being spread onto agar plates overnight allowing population growth. Controls for each dilution without the addition of EVs were also carried out allowing calculation of percentage change following treatment (**Table 4.4)**. Results of 1:100000 dilution was subsequently excluded from the results due to little/no microbial growth. 8 out of 10 of the species treated showed reduction in microbial population, with *Pseudomonas aeruginosa* and *Bacillus subtilis* showing the greatest reduction decreasing by 87.6% and 87.9% respectively.

Table 4.3: Putative proteins identified in SEC purified EVs 5-minute trypsin shave for run times of 30-, 45- and 70-minutes (n = 3). Including transcript identifiers and blast description. Top blast hit was chosen based on lowest E-value and transcripts were ordered by number of unique peptides. (x – indicates identification).

Transcript ID	Blast ID	30-minute runtime	45-minute runtime	70-minute runtime
TR19715|c0_g1_i1	moesin ezrin radixin	x	x	x
TR18070|c0_g1_i1	acid sphingomyelinase-like phosphodiesterase 3a	x	x	x
TR20146|c0_g1_i2	-	x		x
TR9358|c0_g1_i1	Actin-7	x	x	x
TR17099|c2_g1_i1	Tubulin beta chain	x	x	x
TR18542|c0_g1_i1	Tubulin beta chain	x	x	x
TR22003|c1_g6_i1	Tubulin beta-2C chain	x	x	x
TR22003|c1_g4_i3	Tubulin beta chain		x	x
TR23322|c0_g3_i1	Tubulin beta-2C chain	x	x	x
TR22003|c1_g2_i1	Beta tubulin			x
TR22003|c0_g1_i1	Tubulin beta chain			x
TR21569|c0_g5_i1	-	x	x	x
TR21569|c0_g5_i2	-	x	x	x
TR25036|c3_g1_i12	Cathepsin E-		x	x
TR25036|c3_g1_i14	Lysosomal aspartic protease		x	x
TR3846|c0_g1_i1	cathepsin D	x	x	x
TR6048|c0_g1_i1	aspartic ase oryzasin-1-like	x	x	x
TR25036|c3_g1_i1	cathepsin E-A-like	x	x	x
TR25036|c5_g1_i1	renin	x	x	x
TR25036|c0_g1_i1	lysosomal aspartic protease	x		x
TR25036|c3_g1_i2	cathepsin D	x	x	x
TR25036|c3_g1_i8	lysosomal aspartic protease	x	x	x
TR55450|c0_g1_i1	-	x	x	x
TR16856|c0_g1_i1	DM9 domain-containing	x	x	x
TR18939|c0_g1_i1	-	x	x	x
TR17640|c0_g1_i1	-	x	x	x
TR20530|c0_g1_i1	heat shock 90	x	x	x
TR21065|c0_g1_i1	Heat shock 75 mitochondrial			x
TR24356|c0_g1_i3	programmes cell death 6-interacting			x
TR15792|c0_g1_i1	Golgi-associated plant pathogenesis-related 1	x	x	x
TR23254|c0_g1_i1	leucyl aminopeptidase	x	x	x
TR17173|c0_g1_i1	leishmanolysin-like peptidase	x	x	x
TR19239|c0_g1_i1	-	x	x	x
TR12225|c0_g1_i1	Erythrocyte band 7 integral membrane	x	x	x

Table 4.3: *continued.*

TR16040|c0_g1_i1	Lysosomal Pro-X carboxypeptidase precursor	x	x	x
TR18162|c0_g1_i1	liver basic fatty acid binding	x	x	x
TR26002|c2_g1_i1	cytoplasmin type 5	x	x	x
TR33621|c0_g1_i1	-		x	x
TR29071|c0_g1_i1	actin, partial	x	x	x
TR4440|c0_g1_i1	-	x	x	x
TR23598|c0_g2_i1	adenylate kinase 9	x	x	x
TR17877|c2_g2_i1	tubulin alpha-1A chain-like	x	x	x
TR19159|c0_g1_i1	tubulin alpha-1A chain	x	x	x
TR18958|c0_g1_i1	tubulin alpha-1A chain-like		x	x
TR12612|c0_g1_i1	alpha tubulin		x	x
TR21082|c0_g1_i1	Tubulin GTPase domain	x	x	x
TR17328|c0_g1_i1	Na(+) H(+) exchange regulatory cofactor NHE-RF1		x	x
TR20466|c0_g1_i1	Leucine-rich repeat-containing 23	x	x	x
TR9216|c0_g1_i1	tyrosine 3-monooxygenase tryptophan 5-monooxygenase	x	x	x
TR16536|c0_g1_i1	14-3-3 beta alpha-1		x	x
TR17046|c0_g1_i1	14-3-3 epsilon			x
TR20794|c0_g1_i1	phosphoglycerate kinase 1	x	x	x
TR23598|c0_g1_i1	adenylate kinase 9-like	x	x	x
TR20586|c0_g1_i2	regulator of microtubular dynamics 1-like			x
TR13665|c0_g1_i1	calcyphosin isoform X5		x	x
TR19675|c0_g1_i1	radixin isoform X1	x	x	x
TR20928|c0_g1_i1	Cathepsin B			x
TR11284|c0_g1_i1	Histone H4	x	x	x
TR16097|c0_g1_i1	chloride intracellular channel	x	x	x
TR16168|c0_g1_i1	actin depolymerizing factor		x	x
TR15827|c0_g1_i1	Lysosomal protective	x	x	x
TR17138|c0_g1_i1	fatty acid binding brain	x	x	x
TR17367|c0_g1_i1	enolase		x	x
TR19538|c0_g1_i1	Charged multivesicular body		x	x
TR22854|c0_g1_i4	aquaporin-1	x		x
TR15761|c0_g1_i1	lysosomal alpha-glucosidase	x		x
TR20893|c0_g1_i1	methylthioadenosine phosphorylase	x	x	x
TR12782|c0_g1_i1	8 kDa calcium-binding	x	x	x
TR36972|c0_g1_i1	histone H4-like			x

Table 4.3: *continued.*

TR18466|c1_g2_i1	Globin-3		x	x
TR20091|c0_g1_i1	glucose transport	x		x
TR17869|c0_g1_i1	Phospholipase D3	x	x	x
TR15896|c0_g1_i1	calmodium 6			x
TR17164|c0_g1_i1	glyceraldehyde 3-phosphate dehydrogenase		x	x
TR17741|c0_g1_i1	heat shock 70		x	x
TR22803|c1_g1_i2	annexin A11			x
TR3136|c0_g1_i1	-	x		x
TR20643|c0_g1_i1	Annexin A7	x		x
TR17762|c0_g1_i2	lysosomal acid phosphatase	x		x
TR16407|c0_g1_i2	cathepsin D (lysosomal aspartyl protease)			x
TR22152|c0_g1_i1	Hypothetical protein CLF_104825		x	x
TR16514|c0_g1_i1	-			x
TR23279|c0_g1_i1	tubulin alpha testis-specific		x	x
TR18133|c0_g1_i3	CD63 antigen			x

Table 4.4: Population counts following treatment of populations with EVs for 1 hour (TMTC – too many to count).

Dilution	1:1000		1:10000		
Strain	Before Treatment	After Treatment	Before Treatment	After Treatment	% change
1 - *Escherichia coli*	TMTC	TMTC	616	504	-18.18
2 - *Klebsiella pneumoniae*	TMTC	TMTC	700	728	4
3 - *Proteus mirabilis*	TMTC	TMTC	1976	1504	-23.89
4 - *Pseudomonas aeruginosa*	TMTC	TMTC	720	89	-87.64
5 - *Staphylococcus aureus*	TMTC	TMTC	1416	1664	17.51
6 - *Staphylococcus epidermidis*	TMTC	TMTC	364	356	-2.20
7 – *Staphylococcus saprophyticus*	TMTC	TMTC	2136	1712	-19.85
8 - *Enterococcus faecalis*	TMTC	TMTC	198	133	-32.83
9 - *Bacillus subtilis*	TMTC	TMTC	107	13	-87.85
10 - *Candida albicans*	TMTC	TMTC	18	11	-38.90

4.3.7 *C. daubneyi EV biogenesis*

Following proteomic profiling of *C. daubneyi* EVs and investigation of their effect on bacterial species, the mechanisms through which their biogenesis takes place were investigated. Literature was mined in order to resolve a comprehensive list of proteins identified to have roles in EV biogenesis in the closely related trematode *F. hepatica.* These proteins were compared with literature in order to confirm their role in either the ESCRT-dependant or the ESCRT-independent biogenesis pathways. Proteins with confirmed involvement were selected and their sequences in well studied helminth species/human models were obtained from the UniProt database. These sequences were submitted to a local BLAST search against the in-house *C. daubneyi* transcriptome on Bioedit with and E-value of $<E^{-10}$.

In total 89 proteins were identified as having roles in EV biogenesis for both exosome and microvesicle formation, accounting for all currently known pathways through which they can be formed and released. Searched revealed positive identification of homologs for all 89 biogenesis proteins in the transcriptome (**Table 4.5**). Sequences with homology to more than one transcript were selected based on lowest E-value.

Table 4.5: Putative homologs of the EV biogenesis pathways in *C. daubneyi.* Proteins identified in literature were added to the biogenesis work in *F. hepatica* carried out by Cwiklinski *et al*. 2015. Proteins resolved from literature were subject to tBLASTn analysis against the *C.* daubneyi transcriptome identifying homologous sequences.

Protein name	**Uniprot accession**	***C. daubneyi* identifier**	**E Value**
ESCRT dependent pathway components			
ESCRT-0			
HGS	H2KVA9	TR23550|c0_g2_i1	1E-81
STAMBP	H2KPP3	TR23444|c2_g4_i1	2E-98
STAM	G7Y9K6	TR24856|c0_g1_i1	1E-155
ESCRT-I			
TSG101	H2KP02	TR22183|c0_g1_i1	1E-66
VPS28	Q5DGV7	TR16594|c0_g1_i1	3E-91
VPS37	G7YTY4	TR14785|c0_g1_i1	7E-15
MVB12a	H2KR27	TR18657|c0_g1_i1	1E-30
ESCRT-II			
VPS22/SNF8	G7Y7B2	TR17286|c0_g1_i1	1E-97
VPS25	G7YNR9	TR17008|c0_g1_i1	2E-78
VPS36	H2KUN0	TR23092|c0_g1_i1	1E-106
ESCRT-III			
CHMP2A	G7YBN0	TR18914|c0_g1_i1	4E-86
CHMP2B	G7YMJ9	TR24533|c0_g1_i1	1E-91
CHMP6	H2KSF3	TR18646|c0_g1_i1	2E-61
CHMP3	H2KVS7	TR17350|c0_g1_i1	4E-73
CHMP4	H2KVP6	TR22699|c0_g1_i2	1E-66
CHMP5	G7YQI3	TR17234|c0_g1_i1	1E-91
CHMP1a	Q9HD42	TR19538|c0_g1_i1	3E-51
CHMP1b	Q7LBR1	TR17070|c0_g1_i1	2E-61
IST1	G7YUN3	TR19036|c0_g1_i1	1E-87
Vps4-Vta1 complex			
VPS4	H2KR36	TR20248|c0_g1_i1	1E-171
VTA1	G7YED6	TR21759|c0_g1_i1	3E-66
Bro1/ALIX			
ALIX	H2KNH6	TR24356|c0_g1_i3	0
BRO1 domain-containing protein	H2KNG3	TR19613|c1_g1_i1	1E-139
Related compounds			
Syndecan	G7YEG1	TR23157|c0_g1_i1	1E-105
Syntenin 1	G7Y6Z7	TR20161|c0_g1_i2	4E-33
SIMPLE	Q99732	TR13101|c0_g1_i2	3E-24
ESCRT independent pathway components			
Ceramide and lipids			

Lipolytic enzymes			
SMPD2, Neutral sphingomyelinase	O60906	TR24280|c0_g1_i1	1E-37
Acid sphingomyelinase	H2KTZ7	TR18070|c0_g1_i1	1E-113
SMS2, Sphingomyelin synthase 2	H2KQF2	TR18200|c0_g1_i1	1E-127
SphK2, Sphingosine kinase 2	C1LJ82	TR18195|c0_g1_i2	7E-74
PLD, Phospholipase D	G7YNY2	TR25538|c0_g1_i8	0
PLA2, Phospholipase A2	G7YM87	TR24224|c0_g1_i1	1E-175
Phospholipase B-like 2	C1LID9	TR24469|c1_g4_i4	1E-169
Signal transduction			
Flotillin 1	C1LLP3	TR23159|c0_g1_i3	1E-122
Flotillin 2	C7TZR5	TR23768|c0_g1_i1	0
DKG	G7YB49	TR25575|c0_g1_i1	0
Lipids Transport			
ABCA1	G7YFR4	TR24860|c0_g1_i1	0
ABCA3	H2KVB6	TR24860|c0_g1_i1	1E-91
ABCB1	P08183	TR24728|c1_g1_i1	0
MDR1/P-gp	G4VIC6	TR25282|c0_g1_i1	0
Oligosaccharidyl-lipid flippase family	H2KUI4	TR23100|c2_g1_i2	1E-176
Flippase	G7YEB4	TR25392|c0_g1_i1	0
Phospholipase scramblase 2	G7YAS3	TR17446|c0_g1_i11	7E-61
Phospholipase scramblase 3	G7YL60	TR22260|c0_g1_i2	2E-35
Oxysterol binding protein	G7YVH1	TR24801|c1_g1_i4	0
Niemann-Pick C1 protein	G7YQQ4	TR22508|c0_g1_i1	0
Niemann-Pick C2 protein	G7YJT4	TR14799|c0_g1_i1	4E-29
Tetraspanins			
CD63 antigen	G7YRI5		
Tetraspanin CD63-receptor	H2KVE5	TR21067|c0_g1_i2	4E-38
CD9 antigen	G7YQ13	TR20704|c0_g1_i2	4E-93
Tetraspanin 1	G7Y810	TR22166|c0_g1_i1	5E-64
CD81	G7YAH0	TR19451|c0_g1_i1	1E-101
Tspan8	P19075	TR17421|c0_g1_i1	6E-30
CD37	P11049	TR21580|c0_g1_i1	2E-15
CD82	P27701	TR19451|c0_g1_i1	2E-23
CD151	P48509	TR21262|c0_g1_i1	1E-26
Cargo sorting			
Protein sorting			
HSP 70	B1NI98	TR17741|c0_g1_i1	0
HSP90	P07900	TR20530|c0_g1_i1	0
14-3-3 protein	H2KNZ3	TR17046|c0_g1_i1	1E-116
14-3-3 protein B/a-1	Q5FX78	TR9216|c0_g1_i1	1E-93
RNA sorting			
hnRNPA2B1	H2KT13	TR18213|c0_g1_i1	1E-158
Annexin B2	C3VEV0	TR18168|c0_g1_i1	1E-104
Major vault protein	G4V9U9	TR20947|c0_g2_i1	0

Lipid sorting			
Leukotriene-A4 hydrolase	P09960	TR23286|c0_g1_i1	1E-132
Prostaglandin E synthase 3	G7YDD8	TR18874|c0_g3_i1	1E-43
Membrane trafficking and cytoskeleton regulation			
Small GTPases			
RAB27A	H2KNW4	TR23571|c0_g5_i1	1E-123
RAB35	G7Y8S9	TR21595|c0_g1_i2	3E-89
RAB-11	C1L612	TR16663|c0_g1_i1	8E-78
RAB-8A	Q86ET1	TR18583|c0_g1_i1	7E-80
Ras-related protein Ral-A	G7YCE6	TR17790|c0_g1_i1	3E-57
ARF6	P62330	TR21274|c0_g1_i1	6E-79
Ras-like GTP-binding protein Rho1	S4PGT9	TR19491|c0_g1_i1	1E-91
TBC domain family member 20	H2KNS4	TR21689|c0_g1_i1	0
SNAREs			
Syntaxin	G7YP44	TR23441|c1_g2_i1	1E-42
Synaptobrevin homolog YKT6	H2KTY8	TR19416|c0_g1_i1	1E-84
Synaptotagmin	H2KPI2	TR23597|c0_g1_i1	8E-91
Synaptosomal-associated protein	H2KRN3	TR19698|c0_g1_i1	5E-86
VAMP7	G7YB99	TR20542|c0_g1_i1	1E-48
Proton pumps			
V-type H+-transporting ATPase subunit A	H2KPW7	TR21854|c0_g1_i1	0
V-ATPase VHA5	G5EEK9	TR20462|c0_g1_i3	1E-175
H+-transporting ATPase	H2KTS1	TR20462|c0_g1_i1	0
ATPase H+ transporting, lysosomal accessory protein 1	C1LMZ1	TR18290|c0_g1_i1	7E-39
Vacuolar H+ ATPase	C1LDA3	TR16991|c0_g1_i1	1E-158
Cytoskeleton regulation, adhesion, membrane fusion and repair			
Calpain	P27730	TR24050|c0_g1_i2	0
Gelsolin	C1LDA7	TR21445|c0_g1_i2	1E-132
Myosin light chain kinase	H2KUH9	TR25975|c0_g1_i1	4E-65
ERK	H2KSY3	TR23314|c1_g1_i1	1E-166
Annexin B22	C4QH88	TR20643|c0_g1_i1	1E-122
Annexin	G7Y5I1	TR20349|c0_g1_i1	1E-139
Annexin	H2KP46	TR22803|c1_g1_i1	1E-138
Thrombospondin	H2KTG5	TR23932|c0_g1_i1	1E-154
Vesicle-fusing ATPase	G7Y509	TR25314|c0_g1_i1	1E-45
Myoferlin	G7YHB9	TR25557|c0_g3_i2	1E-179
Otoferlin	G7YR21	TR25557|c0_g3_i3	1E-68

4.4 DISCUSSION

This chapter aimed to positively identify and characterise EVs in *C. daubneyi* excretory-secretory (ES) products. Upon positive identification, the mechanisms behind their release as well as their surface protein interactions with bacterial populations were also investigated.

Identification of EVs in *C. daubneyi* ES products

Extracellular vesicles were successfully isolated from *C. daubneyi* ES products through both differential centrifugation (DC) and size exclusion chromatography (SEC). The fractions isolated using both techniques were confirmed to contain EV populations through transmission electron microscopy (TEM) - identifying membrane bound vesicles varying from 30-200 µm in size. TEM imaging also identified contamination of samples (debris) regardless of a 0.2 µm filtration step for bacterial removal. Due to the absence of ruptured EVs during TEM analysis these samples were deemed viable for downstream analysis.

DC is one of the most widely used EV isolation techniques accounting for 81% of purifications in published studies (Gardiner *et al.*, 2016). Despite its widespread use, isolation through DC can be problematic due to the high level of aggregation observed leading to mixed populations of EVs with varying phenotypes and morphologies (Linares *et al.*, 2015). Aggregation was observed in the EVs isolated through DC in this study, but not in the samples collected through SEC. Asides from aggregation there were no notable morphological differences between purification techniques with both fractions containing an array of both smaller and larger vesicles. TEM analysis has been found to overestimate level of aggregation due to the retention of larger particles whilst smaller particles are able to pass through the grid (Linares *et al.*, 2015).

Due to the presence of aggregation, SEC purified samples were utilised in the trypsin shave and antimicrobial activity studies due to their improved functionality, with DC known to have an impact on surface signalling molecules affecting their ability to activate and subsequently be up taken by recipient cells that could affect the reliability of their use in experimentation (Mol *et al.,* 2017), whilst SEC has been found to retain EVs in a functional state (Davis *et al.,* 2019).

Proteomic investigation of *C. daubneyi* EVs

Utilisation of the recently reported adult *C. daubneyi* transcriptome (Huson *et al.,* 2018) allowed a comprehensive proteomic characterisation of the adult helminth's membrane bound vesicle secretions, leading to identification of 378 proteins consistent across three biological replicates. Comparison with a resolved eukaryote EV proteome identified a number of proteins in common including, tetraspanins (TR20913|c0_g1_i1, TR22166|c0_g1_i1, TR22094|c0_g1_i1 and TR22869|c0_g1_i12), Heat shock proteins (TR17741|c0_g1_i1 and TR20530|c0_g1_i1), Annexins (TR22803|c1_g1_i2, TR20643|c0_g1_i1 and TR17648|c0_g1_i1), as well as EV associated cytoskeletal proteins such as Actin (TR9358|c0_g1_i1, TR17779|c0_g1_i1 and TR28482|c0_g1_i1) and Ezrin (TR19715|c0_g1_i1) as well as proteins involved in metabolic processes such as enolase (TR17367|c0_g1_i1, TR24268|c0_g1_i1, TR24268|c0_g2_i1 and TR19628|c0_g2_i1), Peroxidases (TR17193|c0_g1_i1 and TR12513|c0_g1_i1) and pyruvate kinases (TR21788|c0_g1_i1) (Choi *et al.,* 2013; Nowacki *et al.,* 2015). The consistency in proteins with confirmed EV proteomes further supports the identification of the membrane bound vesicles by TEM imaging as EVs, suggesting the *C. daubneyi* secretome is more complex than previously thought (Huson *et al.,* 2017).

Protein cargo packaged into EV prior to their release is dependent upon cellular source and release cell associated activity (Simons & Raposo, 2009). Consistent with closely related trematode *F. hepatica,* rumen fluke EVs returned a large quantity of proteases and peptidases including Xaa-pro peptidase, cathepsins and metalloproteases that have not been identified in the EVs of several trematode species such as *E. caproni*, *S. mansoni* and *D. dendriticum.* Differences in protein cargo packaged between species could be due to their residency within the definitive host but could also be attributed to lifecycle stage at time of release (Nowacki *et al.,* 2015). In order to further investigate the variability in proteins packaged and their roles in establishment a key area of research could be into the hypothetical and 'no-blast' results returned. In total, 14.2% of the proteins identified represented these undefined proteins and their further investigation could allow insight into infection, migration and successful establishment of infection as well as possible drug and vaccine targets (Dalton *et al.*, 2003).

Exosomes and microvesicles produced by *F. hepatica* have been estimated to contain 12.2% of total protein secreted, although this is a relatively low percentage EVs contain a range of immunomodulatory proteins that would have a great effect on the host immune response (Cwiklinski *et al.,* 2015). *F. hepatica* secretions have been found to contain a plethora of molecules found to be internalised by host cells involved in immunomodulation leading to a TH2-mediated environment that is favourable for parasite establishment (Dalton *et al.,* 2003). Consistent with studies of *F. hepatica*, the *C. daubneyi* EV proteome contained fatty-acid binding proteins, glutathione transferases and cathepsin B known to promote the TH2 response through their immunomodulatory activity (Dowling *et al.,* 2010; Robinson *et al.,* 2011; Donnelly *et*

al., 2010). Damage-associated molecular-pattern molecules (DAMPS) are released into the extracellular environment following damage or death of host cells and have also have the ability to adapt the redox in the extracellular region to mimic that of the internal environment leading to idealistic conditions for pathology (Rubartelli & Lotze, 2007). Homologs to several of these DAMP molecules were found within the proteome including heat shock proteins (HSPs) and annexins. DAMP homologs in helminth species can manipulate the immune response from the host that could regulate the host environment preventing the inciting of an unfavourable immune response (Robinson *et al.,* 2010). Also identified in this proteome were three members of the GST superfamily, which are investigated further in **Chapter 5**.

Species ID for each of the top 100 blast results was carried out identifying the top 10 species to which the EV proteome protein sequences matched. Unsurprisingly, out of the top 10 species returned from blast, 8 of them were closely related helminth species accounting for 80.1% of blast results. The 378 sequences identified belonged to a total of 58 species, however many of them only appeared once throughout the proteome analysis with low E-values and percentage coverage. When considering all blast results returned a total of 761 species were identified to which sequences shared homology with the transcripts identified during MS analysis, with many proteins showing homology to more than one helminth species as well as with unrelated species.

Each of the top 100 EV proteins quantified by unique peptides were categorised by their molecular function, 10 categories were devised in order to sort the proteins as previously described by Cwiklinski *et al.,* 2015. The greatest number of proteins accounting for 34% of the top 100 proteins fell into the ‘Other’ category. This is likely

due to the number of uncharacterised and hypothetical proteins returned from the BLAST search as well as the lack of genomic data currently available for *C. daubneyi.* Due to the nature of transcriptomic data any proteins unique to this parasite will not be identified and those with homology to other helminths with little genomic data will have been returned as uncharacterised or hypothetical. The greatest number following this was cytoskeletal proteins accounting for 24% of the total top 100, followed by proteases at 14%. As with previous trematode studies, the presence of uncharacterised proteins allows the hypothesis that they could be novel sequences with potential roles in parasite pathogenesis. Identification of uncharacterised proteins that have no homology to known sequences provides an assortment of possible research avenues into potential future control and interventions of infection (Mulvenna *et al.,* 2010).

Gene Ontology (GO) allows classification and study of enriched terms in three defined groups – Molecular function, Biological process and Cellular components on a hierarchal basis, allowing identification of functional relationships between proteins present in the EVs (Zeeberg *et al.,* 2003; Kim *et al.,* 2013). Gene Ontology has recently been investigated in relation to EV communications due to identification of their increasing importance (Gézsi *et al.,* 2019). Due to the importance of EVs becoming more apparent with increased levels of research, cellular component ontology has now been extended to include metadata including that of EVs allowing their annotations to be included in the databases (The Gene Ontology Consortium; Cheung *et al.,* 2016). GO analysis was utilised to categorise the proteins identified in the EV proteome by functionality. GO terms involved with the plasma membrane were enriched as would be expected due to mechanisms of vesicle biogenesis. Each protein sequence resolved was subject to Gene Ontology analysis, with many of the proteins classified into more

than one gene ontology classification. GO terms associated with EV biogenesis (GO:0140112, GO:0097734 and GO:0016050) all had protein sequences assigned to them – suggesting the presence of biogenesis proteins packaged within the vesicles themselves.

EV trypsin shave and microbial interactions

Following resolution of the total proteome the surface exposed proteins of EVs in *C. daubneyi* were investigated. Numerous helminth parasites have been found to secrete EVs capable of modulation of host immune cells through internalisation (Eichenberger *et al.*, 2018). Packaging of proteins and RNAs that are advantageous as effector molecules protects them from degradation that would take place if they were secreted directly into the extracellular space and so allows them to be effectively delivered to sites distant from their release point (Torre-Escudero *et al.*, 2019). Recipient host cells uptake mechanisms are not well understood with debate over the possibility of it being a mediated or passive process. The lack of knowledge could be due to the variation in proteins expressed on the surface of EVs that the host cells encounter, with studies showing internalisation being influenced by surface proteins and their post translational modifications (PTMs), leading to increased interest in their research (Mulcahy *et al.*, 2014). Generally, EV cargo proteins have been well characterised with many markers known to be used as identifiers of EV (such as the ExoCarta database), little research has been carried out on the molecules that are present on the surface of EVs (de la Torro *et al.*, 2018). It is likely that surface proteins directly interact with host cell receptors and so their investigation could identify possible targets for preventing EV-host cell interactions and so preventing delivery of immunomodulatory molecules.

Trypsin shaving of the surface of *C. daubneyi* EVs returned a total of 86 proteins with the results of both the EV shave and whole EV preparations returning a variety of well-known exosomal markers such as heat shock protein 70 and members of the tetraspanin family as defined by the Exocarta database (http://www.exocarta.com). Several membrane channels and transporters were identified including ATPase, V-type H+- transporting ATPase, phospholipase and glucose transporters. As with the cargo proteins, there were numerous unclassified proteins resolved in this investigation that could be key components in EV adhesion to target cells and subsequent internalisation and delivery of immunomodulatory material. Membrane associated proteins such as Annexins and tegumental proteins were also identified.

Following resolution of the proteins present on the surface of EVs, their potential antimicrobial properties were investigated on a range of bacterial species. Both helminths and bacterial species within the gut have been identified as having strong immunomodulatory effects on the mammalian host, with a variety of studies showing helminths effect on the microbiota and the microbiotas effect on helminth successful establishment (Reynolds *et al.,* 2015). Previous studies in helminth species have shown alterations to bacterial populations within the gut microbiota, with infection leading to regulation of bacterial species (Su *et al.,* 2017). Regulation of bacterial species is important in helminth infection due to the ability of certain species to elicit the host immune response favourable for helminth establishment and survival (Reynolds *et al.,* 2015).

The study into EVs effect on bacterial organisms encompassed ten bacterial species including two model organisms, one gram positive, *B. subtilis* and one-gram negative,

E. coli. EVs showed antimicrobial activity against eight out of the ten organisms to which they were treated. Both model organisms in this study showed a reduction in colony growth following treatment allowing hypothesis that *C. daubneyi* EVs released into the rumen regulate the natural gut microbiota, making it more favourable for their survival allowing successful establishment of infection. Helminth infections have previously been identified as capable of affecting the microbiota structure leading to modifications in abundance of microbial populations as well as impacting directly on various KEGG pathways (Li *et al.,* 2016). Whilst changes to the gut microbiota in the presence of helminth infection has been noted the effects on structure and function remain unstudied (Li *et al.,* 2016). It is not fully understood how helminth infections modulate the mucosal immune response; however, it is hypothesised this could be achieved through alteration of the microbiota allowing favourable conditions in which proinflammatory cytokines are supressed preventing chronic inflammation and allowing successful long-term infection (Weinstock & Elliot, 2009; Walk *et al.,* 2010). Their ability to enhance populations capable of eliciting their desired TH2 immune response could be the mechanism through which establishment and evasion of the hosts immune system is achieved (Cattadori *et al.,* 2016).

Identification of *C. daubneyi* ability to alter the microbiome and so the host immune response could be beneficial in the potential development of immunotherapies that are being investigated in the control of inflammatory diseases (Mishra *et al.,* 2014). A full antimicrobial analysis of all EV proteins characterised would be beneficial as EVs are known to bind to targets in order to become internalised and release their contents and so these may also be influencing the EVs themselves as well as elucidating

mechanisms through which EVs released by *C. daubneyi* manipulate the host microbiota leading to conditions favourable for long term establishment.

Transcriptomic analysis of EV Biogenesis pathways

Following positive identification and characterisation of EVs, the biogenesis mechanisms through which they are formed and released was investigated. A total of 89 protein sequences representing both the ESCRT-dependent (ESCRT-0, -I, -II and -III) and ESCRT-independent pathways (via tetraspanins or various lipid-related enzymes) were identified in literature and subject to local Blast analysis against the in-house transcriptome to identify their presence in *C. daubneyi* and elucidate the mechanisms utilised in their EV formation. As well as proteins involved in these pathways, further proteins such as AAA-ATPase VPS4 involved in the abscission of ILV's into the MVB lumen were also investigated. All 95 of the key biogenesis regulator sequences submitted to local blast returned homologs in the *C. daubneyi* transcriptome whilst *F. hepatica* identified 7 sequences with no homology to any sequences in the genome (Cwiklinski *et al.,* 2015). Based on these results it's apparent that EV biogenesis in adult fluke occurs via both the ESCRT-dependent and ESCRT-independent pathways. Here we have identified that *C. daubneyi* synthesises all the relevant protein components required for exosome and microvesicle biogenesis via all known pathways currently detailed in literature (Niel *et al.,* 2018). These results are in keeping with many studies detailing that biogenesis of microvesicles and exosomes can happen simultaneously with the ratio of each produced controlled by cell type and its status (resting/stimulated) leading to their production (Shen *et al.,* 2011; Trajkovic *et al.,* 2008; Cocucci *et al.,* 2009).

Identification of these biogenesis proteins in turn identifies possible targets for inhibitory compounds that could facilitate parasite control. Prevention of EV formation could inhibit delivery of immunomodulatory molecules that mediate the immune response leading to long term infections unable to be established successfully (Torre-Escurado *et al.,* 2016; Coakley *et al.,* 2017). Targeting of several of the key regulators of biogenesis including sphingomyelinase, ALIX and GTPases through either RNA interference or inhibitory chemicals has been tested in mammalian cells and showed a significant decrease in EV release. Prevention of release using these mechanisms has been noted as an effective mechanism in cancer studies and so could be a key approach to parasite control in the future (Andaloussi *et al.,* 2013). As EVs also play a crucial role in parasite migration, inhibition of their formation in the juvenile parasite may lead to them not reaching the rumen in which they would normally develop into adult fluke and produce eggs preventing the life cycle from continuing (Cwiklinski *et al.,* 2015). Development of a technique that allows selective inhibition of biogenesis pathways could also allow delivery of immunomodulatory molecules to host tissue with the potential to lead to removal of the infection through the host's immune response that is normally suppressed (Torro-Escuardo *et al.,* 2016).

4.5 SUMMARY AND FUTURE RESEARCH

This investigation confirmed the presence EVs in *C. daubneyi* ES products (Huson *et al.*, 2018). EVs have recently gained a large amount of attention in helminths through their ability to be selectively packaged allowing delivery of specific bioactive molecules to target cells. The recent publication of the adult *C. daubneyi* transcriptome as well as developments in omics technologies allowed an in-depth analysis of their EV protein composition as well as investigation into their interaction with bacterial populations and their mechanisms of release. Elucidation of their internalised and surface proteins as well as their biogenesis highlight many further areas of research into potential treatment and vaccination options, with EVs highlighted as a potential mechanism through which the immune response can be tuned in order to supress helminths immunomodulatory action (Zakeri *et al.*, 2018).

CHAPTER 5.

INVESTIGATION INTO THE DETOXIFICATION CAPACITY OF *CALICOPHORON DAUBNEYI*

5.1 INTRODUCTION

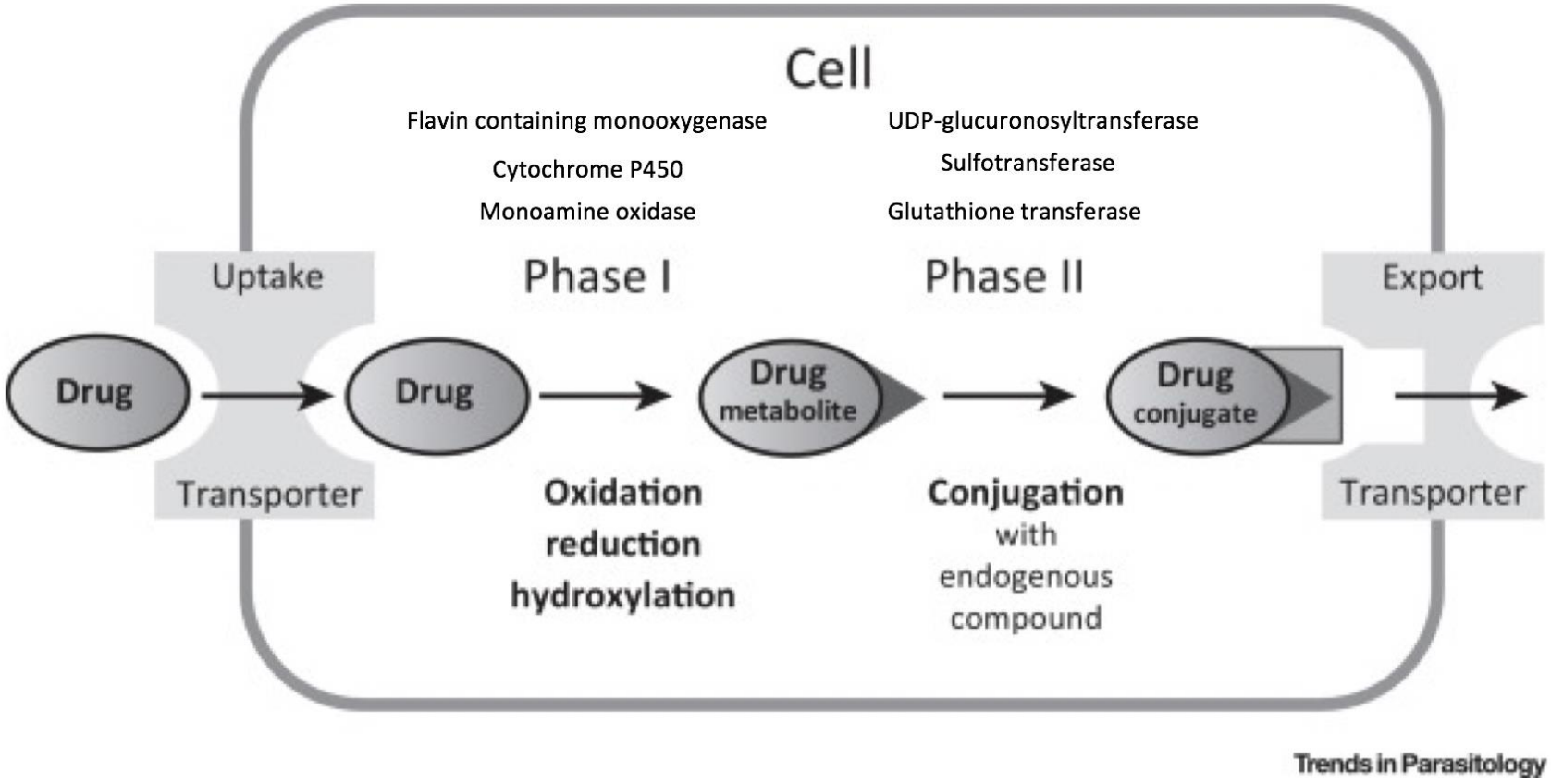
5.1.1 Detoxification enzymes in helminths

Helminths have developed an array of mechanisms allowing their protection against the effects of anthelmintics which in turn facilitate the development of resistance to these compounds that is now recognised as a worldwide problem (Cvilink *et al.,* 2009). To date a number of mechanisms involved in the development of drug resistance have been identified in helminth species, however there are thought to be many more currently undiscovered (Cvilink *et al.,* 2009). A key mechanism leading to development of drug-resistance in helminths is the action of xenobiotic detoxification, with repeated exploitation of xenobiotic metabolising enzymes (XMEs) following contact with anthelminthics leading to induction of biotransformation enzymes and transporters as a means of defence which in turn facilitates development of resistance to these compounds (Cvilink *et al.,* 2008; Kerboeuf *et al.,* 2003).

Anthelmintic resistance has been attributed to target site changes and drug-efflux pathway alterations, with the use of reduction, hydrolysis and conjugation described for many years acknowledging the role of enzymes and transporters in protecting the organism of the negative effects of xenobiotics (Kerboeuf *et al.,* 2003; Sangster, 1996; Munir & Barrett, 1985). XMEs have been studied in a multitude of helminth species, however, due to its status as a newly emerging parasite in Western Europe, there have currently been no studies into the potential detoxification mechanisms that could be utilised by *C. daubneyi,* likely due to no documented reports of resistance due to no licensed treatment options. With drug resistance in parasitic helminths increasing it is important to understand species specific detoxification mechanisms in order allow

effective pharmacotherapy in the future (Cvilink *et al.*, 2008). Developments in resistance also highlight the importance of investigating novel biological pathways and alternative treatment options in order to combat helminth infections in the future (Geary *et al.*, 2004; Munguía *et al.*, 2015). This chapter will focus on investigating the phase I and phase II xenobiotic detoxification pathways utilised by *C. daubneyi* (**Figure 5.1**).

Figure 5.1: Metabolism of xenobiotics. Drugs are metabolized following uptake and undergo oxidation, reduction or hydroxylation producing a drug metabolite with a hydrophilic moiety. This metabolite is utilised in the second phase of detoxification through conjugation with endogenous compounds. This conjugate is then excreted through an export transporter (Matouskova *et al.*, 2016).



5.1.2 Developments in transcriptomics

Transcriptomes are defined as the collection of genes actively transcribed in organisms, tissues or cells at the time of sequencing (Oliveira, 2007). In recent years, transcriptomics has seen significant developments in both affordability and accessibility due to expansion in high-throughput sequencing platforms such as microarray technology (Li *et al.,* 2014). In particular, RNAseq transcriptome profiling utilizing next generation sequencing (NGS) has become a cost-efficient alternative to the previously used method of whole genome sequencing (Morozova *et al.,* 2009). Advances in these technologies has allowed the further study of non-model organisms including neglected parasite species for which de novo assembly was not previously viable (McGettigan, 2013; Choudhary *et al.,* 2015). Transcriptomics combined with downstream bioinformatic analysis allows in-depth mining of produced data sets and has significantly contributed to currently available data on many helminth species (Young *et al.*, 2010a).

5.1.3 Helminth transcriptomics

Genome research of parasitic helminths was first developed through the assembly and subsequent analysis of Expressed Sequence Tags (ESTs) by Fanco *et al.* (1995) with the first parasitic helminth genome sequenced in 2007 (Ghedin *et al.*, 2007). Despite developmets in transcriptomics, EST databases remain the largest source of helminth transcript data avaliable (Garg & Ranganathan, 2012), and have proved invaluable in drug target identification due to the difficulties in producing adequate concentrations of homogeneous material from parasites that would be required for effective genomic sequencing (Doyle *et al.,* 2010). The first transcriptomics investigation into a neglected helminth parasite was carried out by Young *et al.* (2010b) utilizing NGS

followed by bioinformatic interrogation. Such datasets act as a foundation for further developments in functional genomics allowing the molecular study of metabolic pathways as well as parasite development and reproduction (Young *et al.,* 2010a). To date, only a few parasitic flatworms have fully annotated genomes including *Schistosoma mansoni, Schistosoma japonicum, Clonorchis sinensis* and *Fasciola hepatica* (Zhou *et al.,* 2009; Berriman *et al.,* 2009; Wang *et al.,* 2011; Cwiklinski *et al.,* 2015) all of which are of either great health impact or economic importance. Despite the lack of genomic data for many species, expressed sequence tag (EST) and transcriptomic analysis have contributed greatly to the field of parasitology through production of high throughput and functionally relevant nucleotide sequences allowing in-depth analysis of many helminths host-parasite relationships, establishment and immune evasion through referencing of proteomic data obtained through gel electrophoresis and subsequent LC-MS/MS (Hewitson *et al.,* 2008; Anderson *et al.,* 2015; Robledo *et al.,* 2014; Zhou *et al.,* 2016).

This method has proven effective in identifying and classifying a large proportion of proteins present in the absence of a resolved genome, allowing comparative studies between both species and life cycle stages of these organisms (Robinson *et al.,* 2007; Robinson *et al.,* 2009; Robinson & Connolly, 2005). When combined with proteomics, the use of sequence alignments and phylogenetics have been utilised in the analysis of specific protein families contributing greatly to understanding of host-parasite interactions, with its use in helminths elucidating potential virulence genes associated with establishment of infection (Robinson *et al.,* 2008; Hacariz *et al.,* 2015). Developments in these technologies have allowed in depth research into neglected parasites with un-sequenced genomes allowing research into potential drug

targets for treatment of infection as well as allowing putative identification of diagnostic targets (Doolan *et al.*, 2014; Zhao *et al.*, 2015).

Quantitative and qualitative transcriptomics have been shown to identify important biological aspects of organisms allowing insight into gene expression levels which could allow discovery of novel drug targets/vaccine candidates at different life cycle stages (Ojopi *et al.*, 2007). Due to current gaps in research in the modes of action of many drugs and the vast increase in drug resistance observed following helminths treatment with anthelmintics means transcriptomic studies allowing derivation of parasite specific metabolic pathways and methods of host interaction which can be manipulated in order to develop possible vaccine and drug targets are an ideal tool for future parasitological investigations (Fitzpatrick *et al.*, 2005; Gobert & Jones, 2008).

As there are currently only several *C. daubneyi* ESTs available, each sequence from the in-house transcriptome hit will need to be subjected to manual blast analysis and characterised through comparison to helminth sequences with annotated data-sets. In this study, bioinformatic interrogation has allowed comprehensive analysis of the *C. daubneyi* transcriptome for detoxification families allowing elucidation of the mechanisms behind xenobiotic detoxification, and methods though which *C. daubneyi* could become resistant to anthelmintics in the future. To date there has been no investigation into any rumen fluke species detoxification pathways. This study accounts for the first in depth transcriptomic study of *C. daubneyi* identifying its major Phase I and Phase II detoxification pathways.

5.1.4 CHAPTER AIMS

- Mining of the in-house *C. daubneyi* transcriptome with model organism detoxification sequences to retrieve all sequences with significant similarity to these families.
- In silico translation of mined sequences in order to carry out functional analysis utilising BLAST and Pfam searches will allow characterisation of each sequence and identification of functional domains.
- Alignment of Phase II detoxification GST sequences and subsequent phylogenetic analysis with representative sequences of each class from model organisms allowing classification of proteins identified.
- Experimental identification of GSTs present in soluble somatic, egg and EV soluble proteomes.
- Mining of **Chapter 3** and **Chapter 4**'s resolved proteomes to identify detoxification families actively translated in *C. daubenyi*.

5.2 METHODS

Unless stated otherwise, all methods were carried out as stated in **Chapter 2**. All solutions were made up using ddH_2O and molecular grade reagents.

5.2.1 Bioinformatic analysis – retrieval of known detoxification sequences

Sequences encoding known detoxification proteins were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/) from both model organisms and genome sequenced helminths. The in-house *C. daubneyi* transcriptome was uploaded to Bioedit and a local blast (tBLASTn) analysis utilised allowing the retrieval of sequences with significant similarity ($<E^{-10}$) to the representative sequences. Retrieved sequences were translated using ExPasy (http://web.expasy.org//translate/), and the appropriate reading frame mined for its respective protein sequence. Protein sequences were subject to NCBI Blast (BlastP) analysis allowing identification of top hits and classification. Following protein classification sequences were subject to Pfam and Interpro searches in order to elucidate conserved functional domains from characterised detoxification families.

Following confirmation of proteins as members of known detoxification families, sequences retrieved from the transcriptome and representative sequences were uploaded to BioEdit and subjected to ClustalW allowing alignment of sequences and identification of conserved regions. ClustalW alignment allowed sequences to be phylogenetically analysed. Phylogenetic neighbour-joining bootstrap trees were produced in MEGA v7.0 allowing visualisation of relationships and homology with known classes.

The *C. daubneyi* transcript was analysed for the presence of proteins from 6 detoxification super families: glutathione transferases (GSTs), cytochrome P450 (CYPs), sulfotransferases (SULTs), flavin-containing monooxygenases (FMOs), monoamine oxygenases (MAOs), and UDP-glucurosyltransferase **(**UDPs). The retrieval and subsequent analysis pipeline utilised is outlined in **Figure 5.2.**

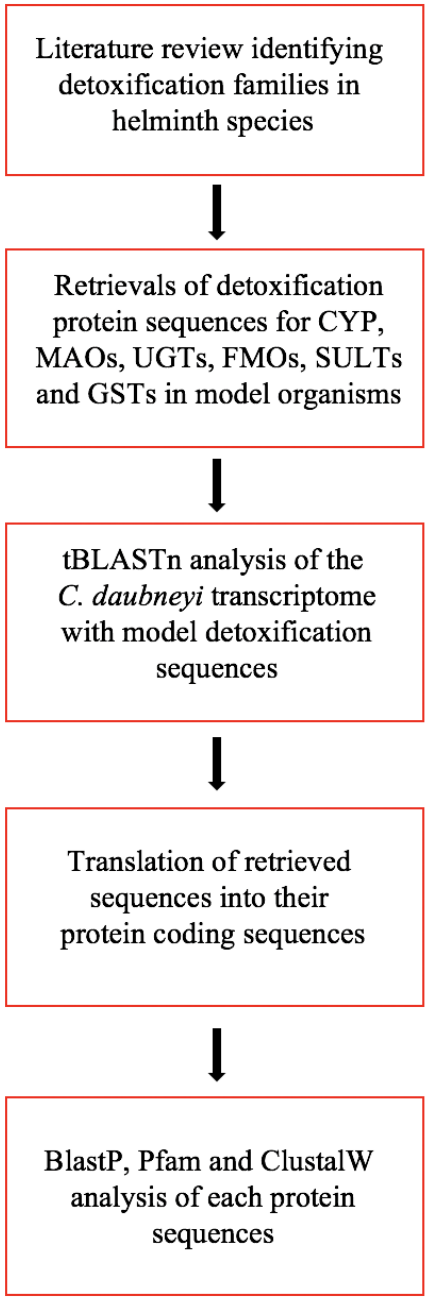


Figure 5.2: Sequence retrieval and subsequent analysis pipeline for detoxification family protein sequences within the *C. daubneyi* transcript.

5.2.2 GST Purification

GSTs were purified from whole rumen fluke tissue, egg and EV fractions through glutathione (GSH)-affinity chromatography following the method described by Simons & Vander Jagt, 1977, with all reagents kept on ice during purification. GSH-agarose (Sigma, U.K) was swelled according to the manufacturer's instructions using ddH_2O before being placed in 1.0 x 5 cm, 4 ml Econo-columns (Bio-Rad, U.K), and allowed to settle resulting in a total bed volume of 1 ml. Lactose was removed from the GSH-agarose through an initial wash with ddH_2O before equilibration with 20 mM potassium phosphate buffer, pH 7.0, 50 mM NaCl for 20 bed volumes at 1 ml/min. Following equilibration samples were added to the column and allowed to flow through by gravity flow, sample was collected and repeated 6 times allowing maximum recovery of GST protein. Flow through was retained and stored. The column was then washed with 20 bed volumes of equilibration buffer allowing removal of non-specifically bound proteins. Bound proteins were then eluted using 5 ml of elution buffer (5mM reduced GSH in 50 mM Tris-HCl, pH 8.0). Elutant was retained and concentrated using Amicon Ultra-4 20 kDa centrifugal filters (Millipore, U.K). Elutants were centrifuged at 3000 rpm for 30 minutes at 4^{o}C and subject to 3 washes in ddH_2O to remove GSH contaminants from samples. The sample was centrifuged until it reached a volume of 250 µl and submitted to Bradford quantification (**Section 2.7)** prior to storage at -20^{o}C for downstream analysis. All washes and flow through were also retained for analysis.

5.2.3 GST Specific Activity Assay

Enzymatic activity was determined pre- and post- purification using the method of Habig *et al.* (1974), through measurement of absorbance change via the process of Glutathione (GSH) conjugation with model substrate CDNB. Briefly, protein samples

were analysed at 25ºC at 340 nm using 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) over a 3-minute period, with a second substrate of 100 mM potassium phosphate pH 6.5, containing 1 mM reduced glutathione using a Cary 50 Bio UV-visible spectrophotometer.

All assays were carried out in triplicate and used to calculate the specific activity of each sample expressed as $min^{-1}mg^{-1}$ through the method of Barrett, 1997 using the equation:

$$\frac{\Delta OD}{\varepsilon \; x \; t} \; x \; V \; x \; L \frac{1}{pr} \; x \; \frac{1}{s} \; x \; 10^{n}$$

ΔOD = change in absorbance over time, t = time (in minutes), ε = extinction coefficient, V = total assay volume (ml), L = path length of the cuvette (cm), pr = protein concentration (mg/mL), s = volume of enzyme extract (ml). n = is dependent on the extinction coefficient (ε) If ε is in $cm^2.M^{-1}$, then *n* = 9, If ε is in $M^{-1}.cm^{-1}$, then *n* = 6, If ε is in $mM^{-1}.cm^{-1}$, then *n* = 3 (Barrett, 1997).

5.2.4 2D Gel electrophoresis

Proteins in this chapter were separated by 2D gel electrophoresis on 2-Dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.4.1 GST sample preparation

Protein concentration were determined through Bradford protein estimation. Specific protein concentrations were then selected, and samples concentrated/diluted to ensure they were in the correct volume (following manufacturers protocol). Samples were then added to 100 µl 1.2 x Buffer Z (9.6 M Urea, 2.4% 3-[(3-Cholamidopropyl)

dimethylammonio]-1-propanesulfonate (CHAPS), 39.6 mM DTT, 0.6% ampholytes pH 3-10, 0.01% bromophenol blue).

5.2.4.2 2-Dimensional SDS-PAGE

Each sample was loaded onto a 7 cm linear pi 3-10, immobilised pH gradient (IPG) strip (Bio-Rad, U.K) in the lane of a rehydration case and flooded with paraffin oil before being left to rehydrate at room temperature overnight. Following rehydration strips were transferred into an IPG focussing tray with excess paraffin oil removed. Wicks were placed at either end on the strips containing 5 µl of ddH_2O and again flooded with paraffin oil. Focussing cases were placed in an Ettan IPGphor system (Amersham Biosciences, U.K) and allowed to undergo isoelectric focussing and allowed to reach 10,000 – 11,000 Volt hours (Vh).

Following focussing, strips were equilibrated in equilibration buffer (50mM Tris-HCl pH 8.8. 6 M Urea, 30% glycerol (v/v) and 2% SDS (w/v)). An initial equilibration for 15 minutes contained 10 mg/ml DTT (Melford, UK), followed by a second equilibration for 15 minutes containing 25 mg/ml IAA (Sigma, UK), both at room temperature. Gels were prepared the same as for 1D electrophoresis **(Section 2.9),** without the addition of a well comb allowing a smooth surface for addition of the IPG strip. To insert the strip 0.5 % w/v agarose in 12.5 mM Tris pH 8.8 was added to the stacking layer and the strip inserted. Wicks were loaded with 3 µl of marker and inserted at each end of the IPG stip. Gels were loaded into the electrophoresis tanks and run as in **Section 2.9**.

5.2.5 2D gel analysis

Following staining, gels were imaging on a GS-800 calibrated densitometer using Quantity One Version 4.6 at 300 d.p.i. (Bio-Rad, UK) gels were stored in 3-4 ml of 1% acetic acid at 4^{o}C prior to mass spectrometry analysis. Images were uploaded to Progenesis PG220 v. 2006 (Nonlinear dynamics Ltd.) for analysis. Gels backgrounds were subtracted using mode of non-spot and spots automatically outlined prior to manual editing. All spots present were outlined and those for contaminants removed. An average gel for each experiment was created by combining the spots of replicate gels (n = 3) and spot volumes normalised, and their respective spot volumes calculated using total spot volume multiplied by total area allowing identification of most abundant spots across each replicate. Average gels presented spots consistent across each replicate as well as those that were only identified on one or two gels. All gel images were then matched through the use of gel warping allowing identification of up/down regulation of proteins between replicates. Each spot was then numbered according to their volumes allowing accurate mass spectrometry preparation.

5.3 RESULTS

5.3.1 *tBLASTn discovery of detoxification families*

In total six detoxification families were analysed for their presence in the *C. daubneyi* transcriptome, three representatives of Phase I detoxification families (CYPs, FMOs and MAO's) and 3 representatives of phase II detoxification families (UGTs, SULTs and GSTs) all of which have been previously characterised in helminth species. Proteins annotated as one of these six families were retrieved from the NCBI and Wormbase databases and for families with multiple classes/sub families, representatives of each were obtained. Each of the protein sequences retrieved were utilised as queries and subjected to tBLASTn searches against the in-house *C. daubneyi* transcriptome. Transcript sequences with significant similarity ($<E^{-10}$) to sequences queried were retrieved and subject to further characterisation. The number of transcript hits retrieved for each detoxification family are outlined in **Table 5.1.**

Table 5.1: Number of transcripts identified in the *C. daubneyi* transcriptome of potential detoxification proteins. In total the transcriptome was mined with model sequences from six detoxification families. Three representative families of Phase I (cytochrome P450s, Flavin-containing monooxygenases and Monoamine oxidases) shaded in grey and three representatives from Phase II (Glutathione transferases, Sulfotransferases and UDP-glucurosyltranseferases). Number of transcript hits represents the number of sequences confirmed to be representatives of these families.

Detoxification Enzyme	**No. of hits in *C. daubneyi* transcript**	**No. of hits in *F.hepatica* genome**
Cytochrome p450 (CYP)	1	1
Flavin-containing Monooxygenase (FMO)	0	0
Monoamine Oxygenase (MAO)	3	3
Glutathione Transferase (GST)	47	10
UDP-glucurosyltranseferase (UGT)	0	0
Sulfotransferase (SULT)	17	2

Of particular interest is the absence of the phase I FMOs and the phase II UGTs, each of which returned no hits to the sequences searched in the transcriptome. No sequences submitted to local blast against the transcript returned any hits even below the significance value of $<E^{-10}$ and due to their absence, there was no further investigation into these families.

5.3.2 Cytochrome P450

In total 4 characterised helminth CYP sequences belonging to helminth species were identified in the NCBI database and their sequences subjected to local blast against the in-house transcriptome. All 4 sequences identified homologs in *C. daubneyi* and were subsequently subjected to Blast, Pfam and Interpro searches to allowing characterisation and identification of conserved family specific domains (**Table 5.2**). Each of the returned sequences were found to contain either the CYP specific domain (PF00067) or the oxidoreductase NAD-binding domain (PF00175), as did each of the resolved helminth CYP sequences utilised in this investigation. Interpro searches of each protein identified the CYP superfamily domain IPR036396 as well as the CYP conserved site IPR017972. In total only one definitive CYP was identified in the *C. daubneyi* transcriptome (TR23398|c0_g2_i1) and following its characterisation its homology to the validated CYPs of helminths was investigated. Interestingly, the other two sequences returning significant similarity to characterised CYPs contained interpro domains for oxidoreductase NAD-binding domains (TR22687|c0_g1_i4 and TR23372|c0_g1_i1), suggesting they are involved in the mechanism through which CYPs work rather than representing CYPs themselves.

Table 5.2: Cytochrome P450 (CYP) contigs identified in the *C. daubneyi* transcriptome. In total 3 contigs returned significant similarity to representative sequences ($<E^{-10}$). Blast identification and top accession hits were recorded as well as Pfam IDs PF00067 (Cytochrome P450 domain) and PF00175 (Oxidoreductase NAD-binding domain).

Contig	E-value	Best hit accession	Species	Description	Pfam ID
TR23398|c0_g2_i1	7.00E-127	AEI26271.1	*Opisthorchis felinus*	Cytochrome P450	PF00067
TR23372|c0_g1_i1	0	TGZ73021.1	*Opisthorchis felinus*	hypothetical protein CRM22_001748	PF00175
TR22687|c0_g1_i4	2.00E-142	THD23105.1	*Fasciola hepatica*	NADPH-ferrihemoprotein reductase	PF00175

5.3.3 Monoamine oxidase

In total 5 sequences were identified to have significant similarity to model MAOs in the *C. daubneyi* transcriptome through tBLASTn analysis. These 5 sequences accounted for 3 proteins, TR24932, TR22272 and TR16773. Retrieved sequences were searched on Pfam identifying domains relating to MAO activity (PF01593), of the five sequences, four were identified to contain domains specific to MAO activity (**Table 5.3**).

5.3.4 Sulfotransferases

In total 17 sequences with significant similarity to known model/helminth SULTs were identified through tBLASTn analysis of the transcriptome. Following isoform removal, a total of 11 proteins representing SULTs were identified. Pfam analysis identified these sequences to contain one of three sulfotransferase domains, PF17784, PF13469 and PF00685 (**Table 5.4**). Out of the 17 sequences three were found to have top blast results to characterised SULTs with significant similarity ($<E^{-20}$), however did not return any results to sulfotransferase domains during Pfam analysis. Of the three sequences that did not identify any domains during Pfam analysis, two were isoforms of which other isoforms of the same protein were identified and did hit SULT domains. TR15065|c0_g1_i1 represents the only proteins with a top SULT blast result with no domain present.

Table 5.3: Monoamine oxidase (MAOs) identified in *C. daubneyi* transcriptome. 5 Transcripts were identified, relating to 3 protein sequences. Resolved sequences were submitted to BLAST and Pfam searches for species identification and Pfam IDs (PF01593 – Amine Oxidase).

Contig	e-value	Best Hit Accession	Species	Description	Pfam ID
TR24932|c0_g1_i1	0	THD25435.1	*Fasciola hepatica*	Lysine-specific histone demethylase	PF01593
TR24932|c0_g1_i2	2E-106	OON17584.1	*Opisthorchis viverrini*	amine oxidase	PF01593
TR22272|c0_g1_i1	0	TPP61473.1	*Fasciola gigantica*	amine oxidase	PF01593
TR16773|c0_g1_i1	0	TPP67665.1	*Fasciola gigantica*	Lysine-specific histone demethylase	PF01593
TR24932|c0_g2_i1	-	-	-	-	-

Table 5.4: Sulfotransferases (SULTs) contigs identified in the *C. daubneyi* transcriptome. In total 17 contigs returned significant homology to representative sequences (<E-10). Blast identification and top accession hits were recorded as well as Pfam ideas PF13469, PF17784, PF00685 and PF03567 (Sulfotransferase families) were also identified.

Contig	E-value	Best Hit Accession	Species	Description	Pfam ID
TR15065|c0_g1_i1	7.00E-27	RJW65123.1	*Clonorchis sinensis*	Protein-tyrosine sulfotransferase 1	-
TR16659|c1_g1_i1	1.00E-21	RTG83172.1	*Schistosoma bovis*	Protein-tyrosine sulfotransferase	-
TR16659|c3_g1_i1	2.00E-80	THD24762.1	*Fasciola hepatica*	Tyrosine sulfotransferase	PF13469
TR18698|c0_g1_i2	1.00E-81	RJW68155.1	*Clonorchis sinensis*	Hypothetical protein CSKR_10301s	PF17784
TR18698|c0_g1_i3	1.00E-81	RJW68155.1	*Clonorchis sinensis*	Hypothetical protein CSKR_10301s	PF17784
TR18698|c0_g1_i4	2.00E-85	RJW68155.1	*Clonorchis sinensis*	Hypothetical protein CSKR_10301s	PF17784
TR18932|c0_g1_i1	7.00E-22	GAA54196.1	*Clonorchis sinensis*	Estone sulfotransferase	PF00685
TR18932|c0_g1_i2	2.00E-132	GAA54196.1	*Clonorchis sinensis*	Estone sulfotransferase	PF00685
TR19038|c0_g1_i1	4.00E-58	TGZ58972.1	*Opisthorchis felineus*	Hypothetical protein CRM22_009325	PF17784
TR19618|c0_g1_i1	4.00E-120	XP_012792458.1	*Schistosoma haematobium*	Hypothetical protein MS3_00827	PF17784
TR21387|c0_g1_i1	7.00E-103	THD21742.1	*Fasciola hepatica*	Heparin sulfate O-sulfotransferase	PF03567
TR22387|c1_g1_i1	1.00E-108	GAA47314.1	*Clonorchis sinensis*	Protein-tyrosine sulfotransferase	PF13469
TR22387|c1_g1_i2	3.00E-50	THD24762.1	*Fasciola hepatica*	Tyrosine sulfotransferase	PF13469
TR2292|c1_g1_i1	1.60E+00	WP_069958027.1	*Magnetovibrio blakemorei*	Insulinase family protein	-
TR23887|c0_g1_i1	7.00E-115	RJW64828.1	*Clonorchis sinensis*	Heparan-sulfate 6-O-sulfotransferase 1-A	PF03567

Table 5.4: *continued.*

TR23887|c0_g1_i2	6.00E-21	TPP56443.1	*Fasciola gigantica*	Heparan-sulfate 6-O-sulfotransferase 1-B	-
TR25897|c0_g1_i1	0.0	THD23554.1	*Fasciola hepatica*	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase	PF00685

5.3.5 Glutathione transferases

Predicted GST sequences were mined from the *C. daubneyi* transcriptome by tBLASTn analysis with 11 representative helminth GST sequences as described by Morphew *et al.*, 2012, as well as GST sequences retrieved from the NCBI database from model organisms, with those with significant similarity ($<E^{-10}$) recorded for further analysis. These nucleotide sequences were translated into their respective amino acid sequences for structural analysis. A Pfam search was carried out allowing identification of conserved domains and characterisation as GSTs as well as division into their respective classes. Two domains allowed the characterization as GST's, one being the C-terminal domain and the second an N-terminal domain. In total bioinformatic interrogation of the transcriptome identified a total of 47 sequences to which there was significant sequence similarity (**Table 5.5**), all of which were positively identified as containing both the N-terminal and C-terminal domains.

The sequences identified were then subject to ClustalW alignment prior to phylogenetic analysis allowing further elucidation of class (**Figure 5.3**). Following phylogenetic analysis of the resolved sequences alone, 36 model GST sequences including representatives of several characterised helminth GST classes were obtained from the NCBI database and combined with the resolved GST sequences and subject to further phylogenetic analysis allowing the grouping of each class allowing visualisation of the class to which each of the newly identified GST sequences belonged (**Figure 5.4**).

Table 5.5: Glutathione transferases (GSTs) contigs identified in the *C. daubneyi* transcriptome. In total 46 contigs returned significant homology to representative sequences (<E^{-10}). Blast identification and top accession hits were recorded as well as Pfam IDs – N-terminal (PF02798 and PF13417) and C-terminal (PF00043, PF14497, and PF13410). Sequences highlighted in red represent those identified below the significance threshold.

Transcript ID	e-value	Accession number	Species	Description	Pfam Id's
TR21421|c0_g1_i1	2.00E-61	OON17888.1	*Opisthorchis viverrini*	glutathione S-transferase protein	N- and C-terminal
TR21421|c0_g1_i2	2.00E-60	OON16509.1	*Opisthorchis viverrini*	glutathione S-transferase protein	N- and C-terminal
TR21421|c0_g1_i3	2.00E-53	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR21421|c0_g1_i4	4.00E-62	OON16509.1	*Opisthorchis viverrini*	glutathione S-transferase protein	N- and C-terminal
TR21421|c0_g1_i5	4.00E-60	OON16509.1	*Opisthorchis viverrini*	glutathione S-transferase protein	N- and C-terminal
TR21421|c0_g1_i6	8.00E-37	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR16211|c0_g1_i1	6.00E-69	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR17879|c0_g1_i1	5.00E-53	RJW65142	*Clonorchis sinensis*	Glutathione S-transferase class-mu 28 kDa isozyme	N- and C-terminal
TR17879|c0_g1_i2	2.00E-50	AAB03573	*Schistosoma japonicum*	glutathione S-transferase protein	N- and C-terminal
TR20023|c1_g1_i1	9.00E-64	OON17888.1	*Opisthorchis viverrini*	glutathione s-transferase protein	N- and C-terminal
TR15503|c0_g1_i1	9.00E-53	RJW65142.1	*Clonorchis sinensis*	Glutathione S-transferase class-mu 28 kDa isozyme	N- and C-terminal

Table 5.5: *continued.*

TR21041|c2_g1_i2	9.00E-32	GAA54849.1	*Clonorchis sinensis*	prostaglandin-H2 D-isomerase	N- and C-terminal
TR22477|c0_g1_i1	9.00E-26	XP_009163004.1	*Opisthorchis viverrini*	hypothetical protein T265_00845	N- and C-terminal
TR22711|c1_g1_i1	4.00E-59	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR22711|c1_g1_i2	6.00E-37	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR16917|c0_g1_i2	5.00E-47	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR16613|c0_g2_i1	5.00E-46	XP_009164764.1	*Opisthorchis viverrini*	hypothetical protein T265_02296	N- and C-terminal
TR47379|c0_g1_i1	3.00E-32	XP_009163005.1	*Opisthorchis viverrini*	hypothetical protein T265_00846	N- and C-terminal
TR22477|c0_g1_i7	1.00E-25	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR22477|c0_g1_i2	1.00E-25	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR22477|c0_g1_i5	1.00E-24	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR21041|c2_g1_i1	6.00E-22	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR22477|c0_g1_i3	4.00E-26	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR21279|c0_g3_i8	1.00E-22	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR22477|c0_g1_i4	7.00E-26	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal

Table 5.5: *continued.*

TR21279|c0_g3_i6	7.00E-23	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR22477|c0_g1_i6	2.00E-25	ABA56496	*Clonorchis sinensis*	glutathione transferase	N- and C-terminal
TR16917|c0_g1_i1	5.00E-22	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR21279|c0_g3_i4	3.00E-22	XP_009163005.1	*Opisthorchis viverrini*	hypothetical protein T265_00846	N- and C-terminal
TR21279|c0_g3_i7	9.00E-21	XP_009163005.1	*Opisthorchis viverrini*	hypothetical protein T265_00846	N- and C-terminal
TR22711|c1_g2_i1	4.00E-04	OON17888.1	*Opisthorchis viverrini*	glutathione S-transferase protein	N- and C-terminal
TR21279|c0_g3_i5	1.00E-21	XP_009163005.1	*Opisthorchis viverrini*	hypothetical protein T265_00846	N- and C-terminal
TR21041|c2_g1_i3	1.00E-21	PIS82752	*Fasciola hepatica*	glutathione S-transferase protein	N- and C-terminal
TR21279|c0_g5_i2	2.00E-28	CAX79577.1	*Schistosoma japonicum*	Glutathione S-Transferase	N- and C-terminal
TR21279|c0_g5_i1	7.00E-29	AAW27835	*Schistosoma japonicum*	SJCHGC01820 protein	N- and C-terminal
TR17112|c0_g1_i1	1.00E-108	AAB46369.3	*Clonorchis sinensis*	putative glutathione transferase	N- and C-terminal
TR15955|c0_g1_i1	2.00E-80	RJW70947	*Clonorchis sinensis*	Glutathione S-transferase Mu	N- and C-terminal
TR21463|c0_g1_i1	3.00E-100	XP_009175615.1	*Clonorchis sinensis*	hypothetical protein T265_10862	N- and C-terminal
TR21463|c0_g1_i4	2.00E-93	RJW67624.1	*Clonorchis sinensis*	putative maleylacetoacetate isomerase	N- and C-terminal

Table 5.5: *continued.*

TR20139|c0_g1_i1	2.00E-37	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20139|c0_g1_i2	2.00E-81	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20139|c0_g1_i3	2.00E-37	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20139|c0_g1_i4	2.00E-38	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20139|c0_g1_i5	2.00E-80	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20139|c0_g1_i6	2.00E-38	ANK78262.1	*Clonorchis sinensis*	glutathione transferase omega-1	N- and C-terminal
TR20132|c0_g1_i1	2.00E-92	QAT98060	*Fasciola hepatica*	glutathione S-transferase omega class 2	N- and C-terminal
TR20132|c0_g1_i2	1.00E-59	QAT98060	*Fasciola hepatica*	glutathione S-transferase omega class 2	N- and C-terminal

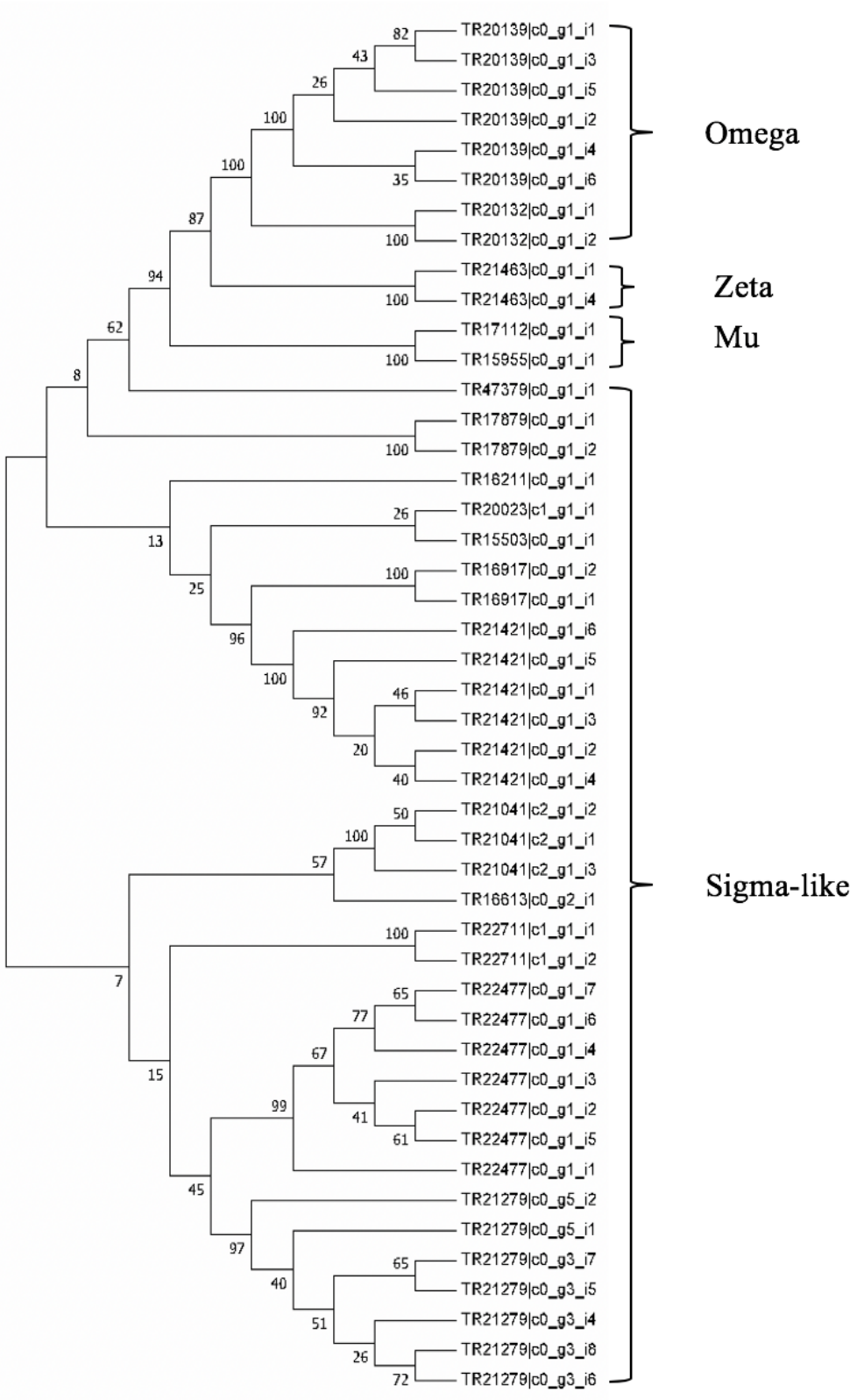


Figure 5.3: Phylogenetic analysis of GST sequences resolved from the *C. daubneyi* transcript. Constructed using a circular neighbour-joining tree following amino acid alignment on Mega 7.0 with 1000 bootstraps and a Poisson correction. Putatively annotated by class through Pfam and Blast analysis.

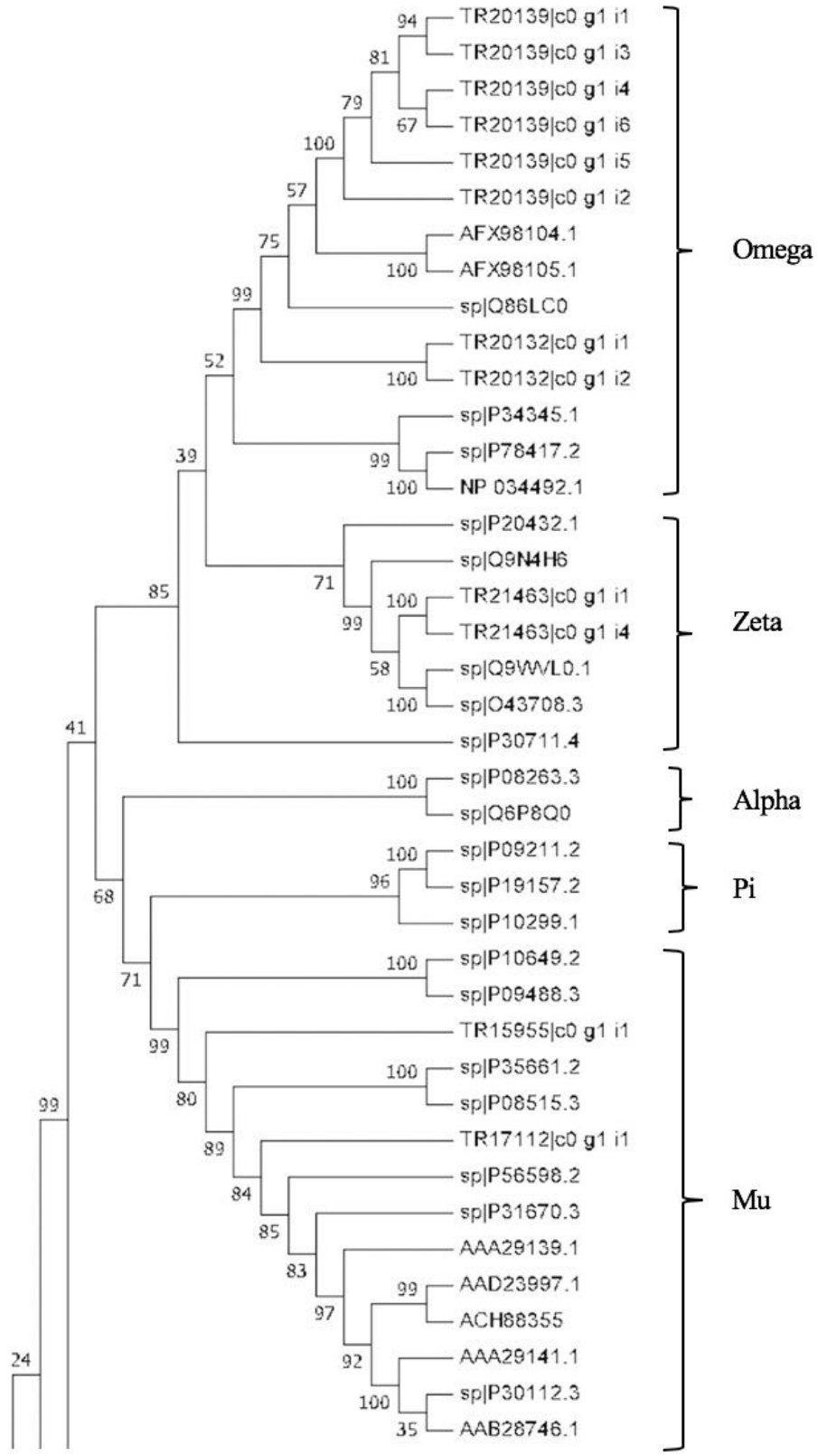


Figure 5.4: Phylogenetic analysis of *C. daubneyi* transcript GST superfamily, including representative characterised members of 6 known classes – Omega, Zeta, Alpha, Pi, Mu and Sigma. Constructed using a circular neighbour-joining tree following amino acid alignment on Mega 7.0 with 1000 bootstraps and a Poisson correction. Sequences accessions from model organisms on NCBI and Transcript identifiers from transcripts resolved at Aberystwyth University. Putatively annotated through Blast, Pfam and phylogeny.

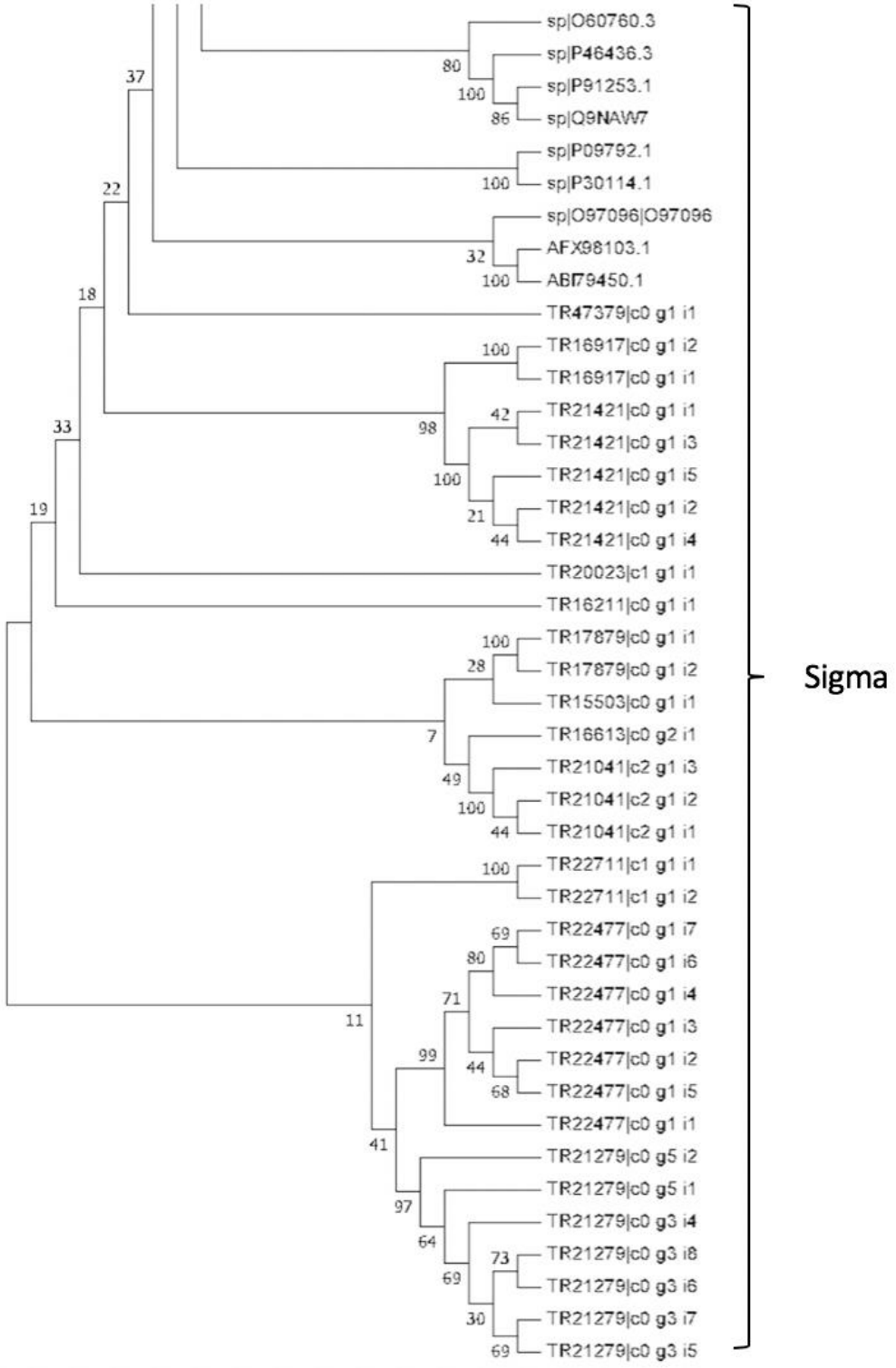


Figure 5.4: *continued.* Phylogenetic analysis of *C. daubneyi* transcript GST superfamily, including representative characterised members of classes – Omega, Zeta, Alpha, Pi, Mu and Sigma. Constructed using a circular neighbour-joining tree following amino acid alignment on Mega 7.0 with 1000 bootstraps and a Poisson correction. Sequences accessions from model organisms on NCBI and Transcript identifiers from transcripts resolved at Aberystwyth University. Putatively annotated through Blast, Pfam and phylogeny.

Following BLAST analysis identifying 47 GST sequences in the *C. daubneyi* transcriptome and subsequent phylogenetic analysis allowing resolution of the class to which each belonged, multiple isoforms were removed, and each remaining protein classified into one of the four GST classes. In total 12 proteins were putatively classified as Sigma-like, 2 as Mu, 1 as Zeta and 2 as Omega (**Table 5.6**).

Table 5.6: Putative classification of GST protein families identified in the *C. daubneyi* transcriptome into their respective classes following NCBI, Pfam and phylogenetic analysis.

Putative GST classifications			
Sigma-like	**Mu**	**Zeta**	**Omega**
TR15503	TR17112	TR21463	TR20139
TR16211	TR15955		TR20132
TR16613			
TR16917			
TR17879			
TR20023			
TR21041			
TR21279			
TR21421			
TR22477			
TR22711			
TR47379			

5.3.5 SDS-PAGE Proteomic Somatic GST

Following bioinformatic interrogation of the transcriptome leading to identification of numerous GST proteins, experimental work was carried out in order to identify GSTs present in the soluble somatic proteome of adult worms. Following sample preparation and resolution on a polyacrylamide gel (**Figure 5.5a**), gels were analysed using Progenesis software (n=3) (**Figure 5.5b**) and each protein present cut and submitted to trypsin digestion prior to LC-MS/MS analysis. In total 31 protein spots were found to be consistent across all three replicates of which 28 proteins were positively identified as GSTs through Blast analysis. All GSTs identified belonged to either sigma-like or mu classes and were interrogated using Pfam analysis. Following removal of isoforms, a total of 7 proteins were identified.

Table 5.7: Total and specific GST activity pre- and post-purification for *C. daubneyi* soluble somatic fraction using CDNB substrate (n = 3). Protein concentrations were determined using the method of Bradford allowing calculation of protein recovery rate.

Sample	Total activity (nmol/min)	Total protein (mg)	Specific Activity (mean S.D) (nmol/min/mg)
Somatic (Pre-purification)	13161.46	9.1	1367.04± 112.10
Somatic (Post-purification)	2488.02	0.21	2764.47±226.70

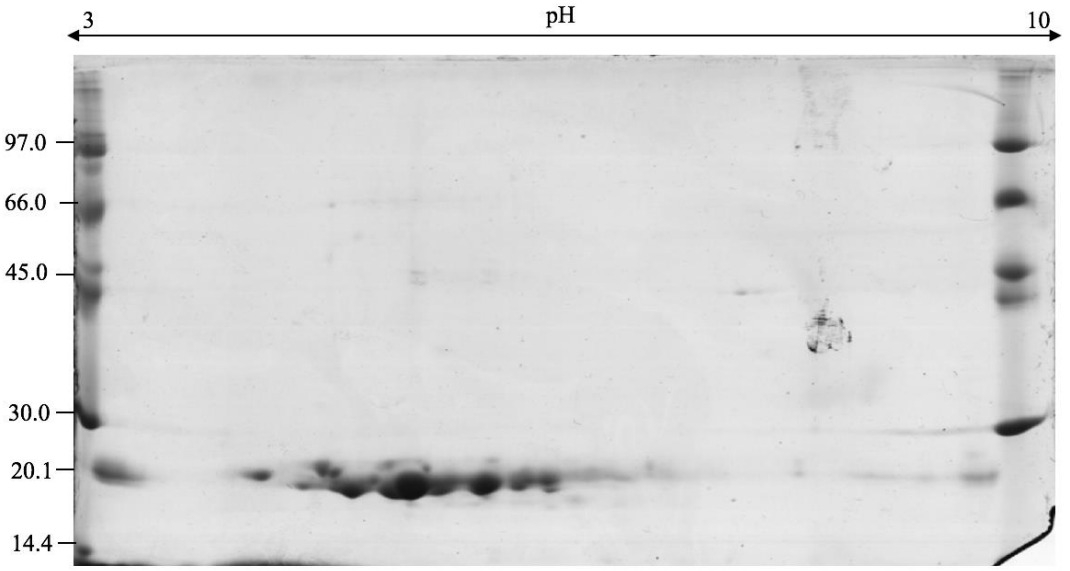


Figure 5.5 (A): Visualization of GST proteins of *Calicophoron daubneyi* adult worms using two-dimensional gel electrophoresis (2-DE): 15 µg of protein was resolved on non-linear IPG strips separate by charge and in the second dimension by molecular weight on 12.5%, 7cm polyacrylamide gels (n=3).

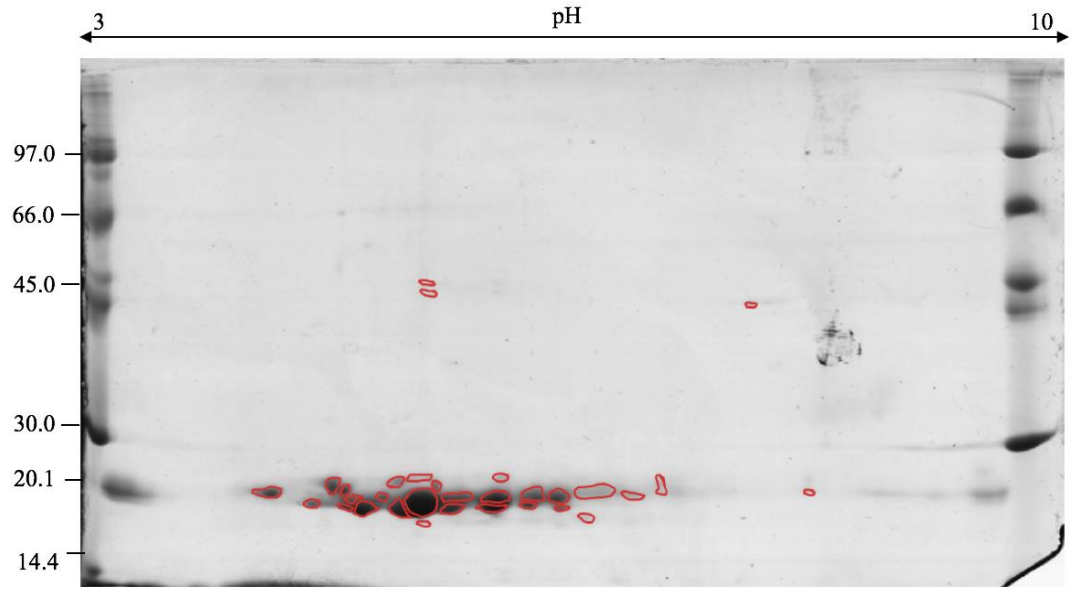


Figure 5.5 (B): Comparative gel analysis following resolution of soluble somatic GST polyacrylamide gels using progenesis software. Each highlighted protein was found to be replicable across all three gels and subsequently subject to LC-MS/MS (n=3).

Table 5.8: Proteins identified following g 2D SDS-PAGE of GSH-purified soluble somatic GST (n=3). Each sequence returned was subject to Blast and Pfam analysis. Only sequences above the significance value of 47 were recorded.

Contig identifier	Protein Hit	Species	Accession number	E value	Pfam Domains
TR15955|c0_g1_i1	Glutathione S- transferase Mu 5	*Clonorchis sinensis*	RJW70947.1	8.98E-79	N- and C-
TR16211|c0_g1_i1	Prostaglandin-H2 D-isomerase	*Fasciola gigantica*	TPP56383.1	4.26E-68	N- and C-
TR17112|c0_g1_i1	Glutathione S- transferase class-mu	*Clonorchis sinensis*	RJW64242.1	7.15E-107	N- and C-
TR21279|c0_g1_i1	Glutathione S-transferase sigma 2	*Meteorus pulchricornis*	QCC89045.1	2.73E-16	N- and C-
TR21279|c0_g3_i1	Prostaglandin-H2 D-isomerase	*Clonorchis sinensis*	GAA54850.1	7.62E-16	N- and C-
TR21279|c0_g3_i2	Prostaglandin-H2 D-isomerase	*Clonorchis sinensis*	GAA54850.1	2.41E-13	N- and C-
TR21279|c0_g3_i3	-	-	-	-	-
TR21279|c0_g3_i4	Glutathione S-transferase	*Fasciola hepatica*	THD22549.1	5.61E-29	N- and C-
TR21279|c0_g3_i5	Glutathione S- transferase class-mu	*Clonorchis sinensis*	RJW69789.1	1.39E-26	N- and C-
TR21279|c0_g3_i6	Prostaglandin-H2 D-isomerase	*Fasciola Gigantica*	TPP56383.1	4.49E-30	N- and C-
TR21279|c0_g3_i7	Glutathione transferase	*Clonorchis sinensis*	ABA56496.1	2.92E-27	N- and C-
TR21279|c0_g3_i8	Glutathione S-transferase	*Fasciola hepatica*	THD22549.1	1.73E-40	N- and C-
TR21279|c0_g4_i1	-	-	-	-	-
TR21279|c0_g4_i2	-	-	-	-	-

Table 5.8: *continued.*

TR21279|c0_g5_i1	Glutathione S-transferase	*Scistosoma japonicum*	CAX79578.1	6.36E-28	N- and C-
TR21279|c0_g5_i2	Glutathione S-transferase	*Scistosoma japonicum*	CAX79578.1	2.25E-28	N- and C-
TR21279|c0_g8_i1	Glutathione S-transferase sigma 2	*Meteorus pulchricornis*	QCC89045.1	1.00E-16	N- and C-
TR22477|c0_g1_i1	Glutathione S-transferase	*Fasciola hepatica*	THD22549.1	3.01E-49	N- and C-
TR22477|c0_g1_i2	Glutathione transferase	*Clonorchis sinensis*	ABA56496.1	1.52E-33	N- and C-
TR22477|c0_g1_i5	Glutathione transferase	*Clonorchis sinensis*	ABA56496.1	4.82E-33	N- and C-
TR22477|c0_g1_i6	Glutathione S-transferase class-mu	*Clonorchis sinensis*	RJW69777.1	2.21E-25	N- and C-
TR22477|c0_g1_i7	Glutathione S-transferase	*Fasciola hepatica*	THD22549.1	2.42E-37	N- and C-
TR22477|c0_g2_i2	Glutathione S-transferase sigma 2	*Meteorus pulchricornis*	QCC89045.1	7.15E-14	N- and C-
TR22477|c0_g2_i3	28 kDa glutathione-S transferase	*Paragonimus westermani*	AAB63382.1	1.25E-17	N- and C-
TR22477|c0_g2_i4	28 kDa glutathione-S transferase	*Paragonimus westermani*	AAB63382.1	2.19E-18	N- and C-
TR22711|c1_g1_i1	Glutathione S-transferase	*Fasciola hepatica*	THD22549.1	4.71E-55	N- and C-
TR22711|c1_g2_i1	Glutathione S-transferase	*Opisthorchis viverrini*	AAL23713.1	3.38E-16	N- and C-
TR33807|c0_g1_i1	-	-	-	-	-

5.3.6 Identification of detoxification proteins in the soluble somatic, egg and EV proteomes

Each of the proteomic profiles detailed in **Chapter 3** and **Chapter 4** accounting for soluble somatic, egg and EV proteins were analysed for the presence of detoxification proteins. Across all three profiles no sequences accounting for MAO, SULTs or CYPs were identified. However, GSTs were identified in all three. In total the somatic proteome identified 19 sequences, the egg proteome 5 and the EV proteome 3 (**Table 5.9)**. The sequences were visualised (**Figure 5.6**), identifying two GST proteins consistent across each of the 3 proteomes, TR16211|c0_g1_i1 and TR17112|c0_g1_i1. Interestingly, the egg proteome identified 2 unique contigs both isoforms of a single protein - TR21041. Whilst the somatic profile returned hits not found in the egg or EV profile, it was also the only proteome to return hits that were not Sigma-like or Mu class identifying 3 hits to Omega class GSTs - TR20132|c0_g1_i1, TR20139|c0_g1_i5 and TR20139|c0_g1_i2 as well as the Zeta class GST - TR21463|c0_g1_i4.

Table 5.9: Interrogation of the resolved somatic, egg and EV proteome (**Chapter 3 & 4**) to identify presence of putative GST proteins. Colour coded by class, Sigma-like (black), Mu (red), Zeta (blue), Omega (green).

Contigs identified		
Egg	**Somatic**	**EV**
TR16211|c0_g1_i1	TR16211|c0_g1_i1	TR21279|c0_g5_i1
TR21041|c2_g1_i2	TR17879|c0_g1_i1	TR17112|c0_g1_i1
TR21041|c2_g1_i3	TR17879|c0_g1_i2	TR16211|c0_g1_i1
TR17112|c0_g1_i1	TR22477|c0_g2_i3	
TR15955|c0_g1_i1	TR22711|c1_g1_i1	
	TR21279|c0_g5_i2	
	TR21279|c0_g3_i6	
	TR21279|c0_g3_i5	
	TR21279|c0_g3_i7	
	TR21279|c0_g3_i8	
	TR21279|c0_g5_i1	
	TR21279|c0_g3_i4	
	TR21279|c0_g5_i2	
	TR17112|c0_g1_i1	
	TR15955|c0_g1_i1	
	TR21463|c0_g1_i4	
	TR20139|c0_g1_i2	
	TR20139|c0_g1_i5	
	TR20132|c0_g1_i1	

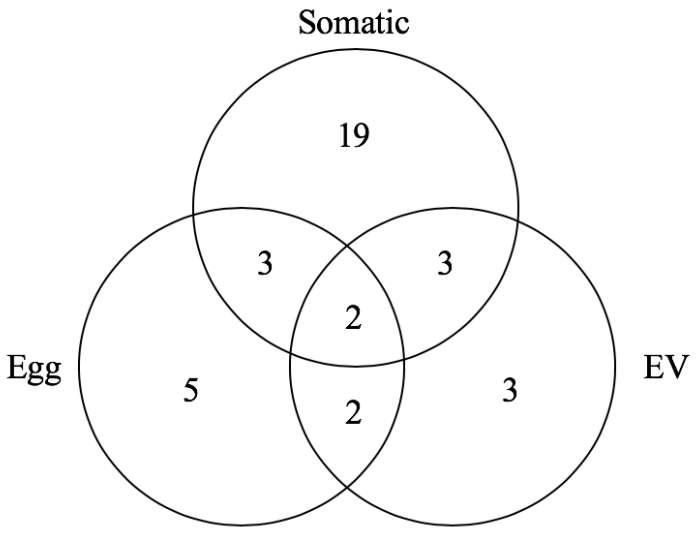


Figure 5.6: Venn diagram representing the quantity of putative GST proteins identified through LC-MS/MS of *C. daubneyi* soluble somatic, egg and EV protein profiles (n=3).

5.4 DISCUSSION

Currently there are few vaccine options available for parasitic helminth infections of livestock and so chemotherapeutic options (anthelmintics) remain the main treatment strategy utilised in both the control of symptoms and elimination of infection (James *et al.,* 2009). Thus, due to the limited treatment options, anthelmintics have been intensively used for intensive livestock production worldwide which has led to the development of resistance to all those licenced for use (Wolstenholme *et al.,* 2004). Owing to the lack of development of new treatment options, understanding the mechanisms through which helminths develop resistance is crucial, allowing the adaptation of current treatments to prevent resistance and surveillance of its development whilst also allowing the elucidation of novel treatment targets (James *et al.*, 2009).

It is well recognised that drug resistance in parasites can be facilitated by the mode of action of XMEs, and bioinformatic studies have allowed progress in understanding their metabolism in different species whilst elucidating potential resistance mechanisms and novel targets for treatment (Matoušková *et al.,* 2016; Lv *et al.*, 2016). Due to its status as a newly emerging disease, it is imperative that potential resistance to anthelmintics in *C. daubneyi* is investigated, especially with the speed at which anthelmintic resistance has previously been observed to different treatment options (**Figure 5.7**). Due to oxyclozanide, albeit off-license in the UK, currently remaining the only viable treatment option, the mechanisms through which rumen fluke may develop resistance and the development of alternative treatments is key.

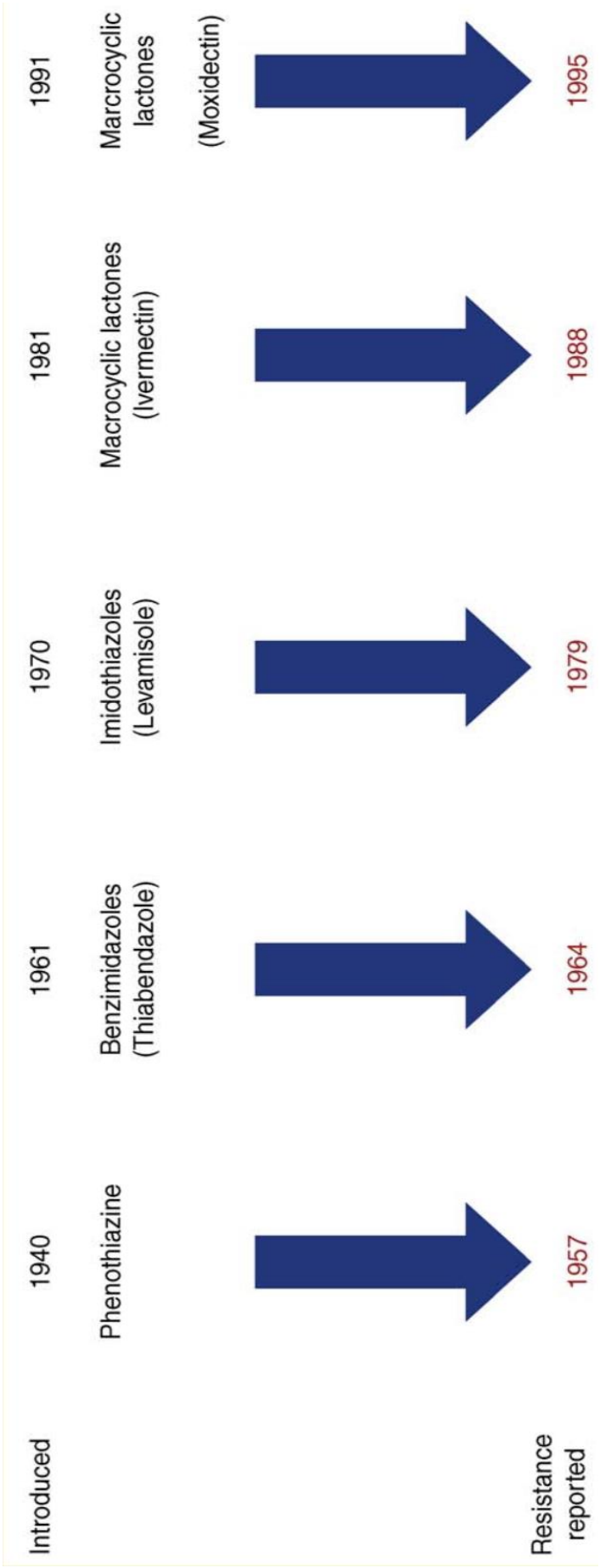


Figure 5.7: Development of resistance to anthelmintics following their initial introduction (James *et al.*, 2009).

5.4.1 Detoxification families

Recent publication of an adult *C. daubneyi* transcriptome allowed a comprehensive analysis of phase I and II detoxification families (XMEs) in this newly emerging parasite. An array of work has been carried out on closely related helminth species identifying many detoxification mechanisms allowing further understanding of the development of anthelmintic resistance (Stuart, PhD Thesis). Of the six detoxification families analysed two did not return any homologs in the transcriptome, these were the phase I flavin-containing monooxygenases (FMOs) and the phase II UDP-glucuronosyltransferases (UGTs). Absence of FMOs in *C. daubneyi* is interesting due to their identification in a transcriptomic study of closely related helminth species, *F. hepatica* (Alvarez *et al.*, 2005), however they have never been identified in genome level studies, and have been noted as absent in an array of parasitic flatworm studies (Pakharukova *et al.*, 2015) whilst UGTs have only been identified in nematode species such as *C. elegans* and *Haemonchus contortus* (Laing *et al.*, 2010; Vokrál *et al.*, 2013). However, the absence of these two detoxification families does not necessarily mean these detoxification pathways are not utilised by *C. daubneyi*, instead these pathways could be utilising a novel form of these proteins not homologous to those searched against the transcriptome, could be left redundant by expansion of other phase I and II detoxification families and so not be required in the biotransformation of xenobiotics or may not be expressed at the time of transcriptomic sequencing as they may be induced through treatment with a xenobiotic leading to their absence in this study. Bioinformatic interrogation of the resolved somatic, egg and EV proteomes (**Chapter 3 & Chapter 4**) returned no hits to CYPs, MAOs or UGTs, however GSTs were found to be highly represented.

5.4.2 Bioinformatic investigation of phase I detoxification families

Cytochrome P450s (CYPs)

CYPs as a family have been found to be responsible for the detoxification of numerous drugs currently in use across species (Ortiz & De Voss, 2002). There are a large number of isoforms within the family with over 200 primary sequences characterised (Benedetti, 2001), however currently only 10 of these isoforms have been identified as playing a role in xenobiotic detoxification (Guengerich, 2005). Early studies hypothesised the absence of CYPs detoxification reactions in parasitic helminths (Precious & Barrett, 1989), however their presence has now been acknowledged in many adult helminths with an expansion of the family identified in free-living species assumed to be due to the toxins the parasites are subject to from the external environment. Recent studies have confirmed CYPs to be an active detoxification mechanism in the closely related trematode *F. hepatica* as well as elucidating its role in the development of its resistance to anthelmintics (Lamenza *et al.,* 2012). Helminth species including *Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium* and *Opisthorchis felinus* have all been identified as only containing a single CYP gene (Nelson *et al.,* 2009). It is thought this reduction in CYPs could represent a simplification in evolutionarily detoxification and a possible reduced requirement for detoxification mechanisms or development of other mechanisms leaving these near redundant (Pakharukova *et al.,* 2012). It has also been hypothesised that the presence of a reduced number of CYPs in several species could indicate a house-keeping role for this gene, with *S. mansoni* single CYP predicted to have a crucial role in egg development and in *H. contortus* a major role in larval stages (Ziniel *et al.,* 2015; Laing *et al.,* 2015).

Bioinformatic interrogation of the *C. daubneyi* transcript identified the presence of CYPs in *C. daubneyi* for the first time. Similar to previous studies in parasitic helminths, this study positively identified a single CYP in the transcriptome with CYP Pfam and interpro domains confirming its characterisation (Nelson *et al.,* 2009). The absence of CYPs in the soluble somatic, egg and EV proteomes is likely due to a housekeeping role in development rather than detoxification and thus left redundant in the adult fluke and so further investigation into CYPs in juvenile *C. daubney*i could be of interest.

Monoamine Oxidases (MAOs)

Research into the involvement of MAOs in xenobiotic detoxification has been largely neglected, mainly due to interest in the research of the Phase I CYPs (Benedetti & Tipton, 1998) as well as MAOs important roles in physiology and behaviour (Sabol *et al.,* 1998). MAOs are able to metabolize amine-containing drugs, however their activity only accounts for the metabolism of 1% of currently marketed drugs compared to the CYPs that account for ~95%. Despite their minimal research MAOs have been identified as a family of enzymes able to catalyse the biotransformation of xenobiotics leading to their excretion from the host (Foti *et al.,* 2016). As a family there has only been two MAOs characterised, MAO-A and MAO-B each of which have been identified as having vastly different substrate specificity (Youdim *et al.,* 2006). Despite their low activity in drug metabolism, developments have been made in the design of drugs that mitigate the effects of MAOs in order to exclude their mechanisms as a means of elimination (Jhee *et al.,* 2001). Interrogation of the transcriptome identified 5 transcripts homologous to characterised MAO sequences accounting for 3 proteins all containing the amino oxidase Pfam domain (PF01593). Identification of

these proteins allows their future characterisation and allowing their inclusion when considering development of potential therapeutics in the future.

5.4.3 Bioinformatic investigation of phase II detoxification families

Sulfotransferases (SULTs)

Local blast analysis of all known helminth SULTs as well as representatives of each known class in humans returned a total of 17 sequences in the *C. daubneyi* transcriptome (**Table 5.4**). The 17 hits returned accounted for 11 proteins following removal of isoforms. Each of the sequences were pulled from the transcript and investigated using Pfam analysis. Across the sequences four Pfam identifiers were resolved, Sulfotransferase 1 (PF00685), Sulfotransferase 2 (PF03567), Sulfotransferase 3 (PF13469) and Sulfotransferase 4 (PF17784).

The full mechanism of SULTs activity during detoxification is still not fully resolved, however it is proposed to function though neutrophilic attack of the sulphur hydroxyl group on 5'-phosphoadenosine-3'-phosphosufate (PAPS) resulting in its transfer to the substrate (Taylor *et al.,* 2017). Previous research into helminth species indicated their inability to carry out sulphate activation and predicted the absence of sulfotransferase activity (Raines & Barrett, 1988). Despite the predicted lack of sulfotransferase activity in helminths, research into *S. mansoni* identified a sulfotransferase gene as the activating enzyme of one of the main treatment options oxamniquine (OXA), with this sulfotransferase activity leading to resistance. Resistance to OXA through sulfotransferase activity was proved through crystallography of its interaction with OXA showing its ability to transfer sulphate groups from PAPS to OXA (Valentim *et al.,* 2013; Pica-Mattoccia *et al*., 2006), as well as confirmation of OXA resistance following RNAi knock down of characterised

Schistosome SULT Smp_089320 (Valentim *et al.,* 2013). This study into sulfotransferase resistance in *S. mansoni* was the first to elucidate a mechanism of an anthelmintic resistance development and allows insight for the future development of drug compounds to treat further helminth infections with the sulfotransferase genes (Cioli *et al.,* 2014) in turn allowing the development and further research into helminths in which sulfotransferase genes have been positively identified. This is the first report of SULTs in *C. daubneyi,* and due to its identified role in development of resistance could prove useful in the future design of drug compounds to treat *C. daubneyi* infections.

Glutathione Transferases (GSTs)

As a superfamily soluble GSTs are a group of antioxidant proteins with many roles in peroxidase, isomerase and thiol transferase activity and are divided into classes based on their structures and specificities (Bae *et al.,* 2016). They have been identified in all studied helminth species and are the main area of focus for a majority of detoxification studies (Brophy & Barrett, 1990; Brophy *et al.,* 2012). Helminth GSTs are presumed to be of great importance with studies showing their presence in an array of trematode species including *C. sinensis* and *Fasciola* species with investigations showing they account for ~4% of the adult soluble protein fraction of closely related trematode *F. hepatica* in which they are also hypothesised to be involved in development of anthelmintic resistance (Chemale *et al.,* 2010; Morphew *et al.,* 2012; Bae *et al.,* 2013; Fernández *et al.,* 2015). In *F. hepatica* four classes of GST have been resolved – Omega (ω), Mu (μ), Sigma (σ) and Zeta (ζ) (Chemale *et al.,* 2006; Morphew *et al.,* 2012). In particular, Sigma class GSTs have gained a lot attention due to their structural properties (Jowsey *et al.,* 2001; Kanaoka *et al.,* 2000), elucidating their

potential to modulate the host immune response (Sommer *et al.,* 2003; Line *et al.,* 2019).

In this study, all of the GST proteins with homology to representative helminth GSTs from defined classes were identified in the transcriptome and subsequently analysed through phylogenetics allowing their classification. In total, 47 GST sequences were found to be homologous to those searched, and isoform removal distinguished 17 GST proteins to be present. Phylogenetic analysis identified 33 of the sequences to be Sigma-like, 2 Mu, 1 Zeta and 2 Omega GSTs to be present. The expansion of sigma-like class GSTs observed in *C. daubneyi* has also been identified in helminths previously, studies of *O. viverrini* and *C. sinensis* also observed an increased number of Sigma/Sigma-like GSTs present. This expansion has been attributed to the parasite's migration through the host in order to reach its definitive residency and sigma class GSTs involvement in parasite migration. Sigma and Mu class GSTs from *F. hepatica* have been researched as a means of serodiagnostics, although it showed high sensitivity it was poor in distinguishing true positive and negative reactions (Aguayo *et al.,* 2018).

Omega GSTs have been identified previously in trematodes that reside within the gut of their definitive host, with Omega class GSTs in *C. elegans* found to have a main role in fighting the oxidative stresses from its gut residency (Burmeister *et al.,* 2008). Omega class GSTs are of particular interest due to their differences in enzymatic properties when compared to other classes such as its GSH-dependent thioltransferase activity and have a proposed role in drug resistance (Board *et al.,* 2000). Omega class GSTs have been identified in several helminth including *S. mansoni*, *Fasciola* and *O. volvulus* (Morphew *et al.,* 2012; Girardini *et al.,* 2002; Liebau *et al.,* 2008), where it

has been identified as acting in the response to oxidative stress (Burmeister *et al.,* 2008) as well as being prevalent in accordance with maturation of the reproductive system (Kim *et al.,* 2019). Identification of the Omega class GST could be beneficial in future treatment of *C. daubneyi* as in other helminth species such as *C. sinensis* Omega class GSTs have been found to play a specific role in the protection of the reproductive system and so could represent an ideal target in halting the parasites lifecycle and preventing reproduction (Kim *et al.,* 2019). Whilst most helminth species have only been found to have a single ortholog of GST-omega, *C. daubneyi* has two which was previously thought to be unique to *C. sinensis* (Kim *et al.,* 2019). Omega class GSTs have been identified as reliable serodiagnostic targets of both *C. sinensis* and *O. viverrini* infections with weak cross-reactivity to other helminth species (Kim *et al.,* 2019). Whilst Sigma and Mu class GSTs did not show sera specificity that would allow them to be a means of diagnostic (Kim *et al.,* 2019). Observation of two GST-Omega orthologous in *C. daubneyi* could be a potential route of future investigation into paramphistome infection.

5.4.4 Proteomic identification of GSTs

All proteins identified within the somatic purified GST sample were resolved in the previous mining of the *C. daubneyi* transcriptome with known GST sequences. Multiple Mu and Sigma class sequences were resolved, however there were no hits to the Zeta and Omega classes previously identified in the transcriptome. 2D-SDS proteomic profiling identified purified proteins to have a molecular weight of 23-28 kDa conducive with investigation of subunits molecular weight (Torres-Rivera & Landa, 2008).

Excretory proteins released by helminths are thought to play a fundamental role in development and immune regulation during infection (Gomez *et al.,* 2015) with their specific expression regulated by environment (Tjalsma *et al.,* 2000). Due to the importance of the secretome (including EVs) and increasing interest into their protein composition in many helminth parasites the presence of GSTs both contained within and on the surface of EVs was investigated. EVs have been found to be a small proportion of the secretome, only accounting for 12% of the proteins released in *F. hepatica* (Cwiklinski *et al.,* 2015). Previous helminth research has shown the packaging of Sigma GST into EVs and their involvement in activation of M2 macrophages facilitating helminth defence (Bae *et al.,* 2016). GSTs have also been identified in the ES products of helminths and were found to be the second most abundant protein identified in the total ES proteome of *C. sinensis* (Bae *et al.,* 2013). ES and membrane GSTs are hypothesised to be involved in as effector molecules against the high level of oxidative molecules they come in direct contact with within the host (Bae *et al.,* 2016). GST abundance in ES leads to interest in possible GSTs secreted in membrane bound vesicles. Experimental GST purification of lysed *C. daubneyi* EVs did not return a significant concentration of protein in order to resolve them on a polyacrylamide gel and submit them to mass spectrometry as were carried out for the somatic fractions. Instead, the egg and EV proteomes resolved (**Chapter 3 + 4**) was analysed for presence of GSTs in both the whole proteome as well as the membrane shave. Interrogation of the whole EV proteome identified 2 GST proteins, TR21729 and TR17112 as well as isoforms of the Sigma class GST TR21279 identified in the EV trypsin shave. Many model organisms such as *E. coli* and a variety of parasitic species who have has their EV proteomes detailed have not identified the GST presence (Marcilla *et al.,* 2012). Interestingly, Sigma class GST identified in EVs

had no predicted transmembrane domains, yet they were positively identified in the trypsin shave of membrane bound proteins. The identification of GST on the membrane of EVs is likely due to GSTs suspected protection role through neutralisation of possible membrane damage from the host immune response through peroxidation (Gobert & Jones, 2008). GSTs presence in *C. daubneyi* EVs could also be of particular importance due to EVs ability to be internalised by host cells and previous trematode studies identifying sigma-class GSTs ability to activate M2 macrophages and so modulate the immune response of the host (Bae *et al.*, 2016).

5.5 SUMMARY AND FURTHER RESEARCH

Utilisation of an '*in silico'* approach allowed prediction of the detoxification enzymes present in *C. daubneyi*. Bioinformatic investigation successfully identified the presence of two phase I detoxification enzymes, CYPs and MAOs, and two, phase II detoxification enzymes, SULTs and GSTs. Surprisingly there was no evidence of FMOs or UGTs in the transcriptome despite their identification in numerous helminth species. Due to lack of genomic data available, this is the first investigation into the potential detoxification capacity of *C. daubneyi*, and discovery allows support for future anthelmintic developments, through understanding of the parasites ability to detoxify anthelmintic candidates. However, it is important to remember that these potential detoxification families will need to be experimentally validated through functional analysis to confirm their roles in xenobiotic detoxification.

CHAPTER 6.

GENERAL DISCUSSION

6.1 INTRODUCTION

The work in this thesis was underpinned by the recent completion of an in-house transcriptome of adult *C. daubneyi* (Huson *et al.*, 2018). This study set out with the aim of uncovering key aspects of the fundamental biology of *C. daubneyi* with an over-arching goal to determine the mechanisms through which establishment of infection is successful, including potential mechanisms of immune evasion and mechanisms through which the parasite is able to detoxify anthelmintic compounds which currently remain unknown. The key motivation for elucidating the fundamental biology of *C. daubneyi* is its status as a newly emerging parasite of ruminant livestock in the UK and Europe, as evident by the large increase in prevalence observed over the last two decades (Foster et al., 2008; Jones *et al.*, 2016). Specifically, the rapid emergence of this parasite throughout the UK has been attributed to movement of livestock and climate change (Jones *et al.*, 2017; Skuce *et al.*, 2013). However, due to the current limited treatment options it is imperative that the basic biology of *C. daubneyi* is further understood to allow the development of future diagnostics and therapies. For example, under current circumstances, with no licenced anthelmintics for treatment and prevalence continuing to rise, developing a detailed understanding of the host-parasite relationship is key in the discovery of anthelmintic candidates whilst minimising the development of resistance. Currently, limited information is available on temperate rumen fluke and the investigations in this thesis have endeavoured to uncover several aspects of its basic biology that will contribute greatly to future studies.

6.2 ADDRESSING THE THESIS AIMS

6.2.1 Characterisation of C. daubneyi adult soluble somatic and egg proteomes

C. daubneyi has recently been acknowledged as the predominant species of rumen fluke in the UK (Jones *et al.,* 2017). However, reports detailing infections of mixed paramphistome species requires the identity of natural infection samples collected, and subsequently utilised in experimentation, to be conclusively confirmed prior to their use (Martinez-Ibeas *et al.,* 2016). Experimental investigation has thus confirmed recent reports of *C. daubneyi* to be the predominant species in the U.K with all samples collected during the current thesis positively identified as *C. daubneyi*.

Paramphistome infections in tropical and sub-tropical regions have been found to cause significant production losses (Ozdal *et al.,* 2010). However, due to its relatively recent introduction into Western Europe, the potential economic impact of *C. daubneyi* in temperate regions is yet to be subject to investigation highlighting the need to gain an understanding of its fundamental biology (Huson *et al.*, 2017). Chapter 3 successfully addressed the thesis aim of resolving the proteome of *C. daubneyi* soluble somatic and egg fractions with both successfully processed allowing their resolution through SDS-PAGE and subsequent LC-MS/MS analysis. A multitude of sequences were resolved from peak spectra data allowing identification of proteins and their function through association with resolved helminths transcripts and annotated genomes. Expansions of specific protein families such as FABPs were acknowledged similarly to studies of many parasitic helminths in which they are currently under experimental validation as vaccine candidates such as fascioliasis and schistosomiasis (Tendler *et al.,* 2015; Ramos *et al.,* 2009) and therefore could also be of interest as potential vaccine candidates to paramphistomosis. Identification of an

extended FABP family is consistent with other helminth species such as *F. hepatica,* where they have been found to promote the TH2 immune response due to their noted immunomodulatory activity allowing successful establishment within the definitive host (Dowling *et al.,* 2010; Robinson *et al.,* 2011). Of particular importance was the resolution of sequences homologous to AC/sHSP's within the transcriptome with numerous sequences also found to be present in the eggs proteomic profile showing significant sequence similarity to AC/sHSP's from multiple helminth species (Moxon *et al.,* 2010). AC/sHSP's have been shown to have high immunogenicity and their presence in *C. daubneyi* eggs suggests the parasites eggs have a role in the host-parasite relationship and interactions with possible roles in successful transmission of infection, as they have been previously described to in several helminth spp. including schistosomes (Moxon *et al.,* 2010; Maizels & McSorley, 2016). An abundance of AC/sHSP's highlights a potential mechanism through which *C. daubneyi* infections successfully establish within the rumen as they provide protection from the harsh environment allowing successful maturation (Li *et al.,* 2009; Caspers *et al.,* 1995).

A further key finding from the proteomic datasets produced is the absence of members of the cathepsin superfamily. Cathepsins have been identified in many helminth species and noted as a key protein involved in successful entry, migration and regulation of the host immune response leading to successful establishment (Cancela *et al.,* 2008; Meemon *et al.,* 2004; Grote *et al.,* 2018). Their absence in these datasets suggest *C. daubneyi* does not utilise the traditional proteins for successful establishment and further investigation into the high number of unidentified and hypothetical proteins could uncover a multitude of proteins unique to *C. daubneyi* establishment.

The data sets elucidated in this chapter can be exploited in future work allowing not only comparative work with further helminth species but also investigation into potential vaccine candidates and markers of infection that may be required if rumen flukes increasing prevalence continues and clinical symptoms are observed in the future. Another potential area of future research of *C. daubenyi* could be investigation of glycosylated proteins, with pathways of glycosylation representing a potential sites of inhibition that could lead to reduced virulence and thus survival within the definitive host, however this area of research in parasitic helminths remains hindered due to the current poor understanding of the mechanisms through which protein glycosylation takes place (McVeigh *et al.,* 2018). Vaccine development has often been inhibited by low efficacy and has been attributed to difficulties in replicating a range of post-translational modification such as glycosylation during production of recombinant proteins (Toet *et al.,* 2014).

It is important to remember that a potential limitation of the proteomic profiling is the stress placed upon the parasite during ex-host culture that may lead to alteration in the proteomic profiles (Morphew *et al.,* 2007). Due to the nature of these samples and their *in vitro* nature, the *in vivo* profile that would be observed is likely to be different. The extent of these potential variations was attempted to be limited by minimising *in vitro* culture periods (Huson, PhD thesis), however, potential *ex vivo* stress could not be completely omitted. A further limitation of this study is mass spectrometry for sequence identification, with proteins of particularly low abundance likely overlooked (Lubec & Afjehi, 2007). Low abundance proteins are of particular importance as they still may have important roles in cellular processes and so further work in resolving and assigning functionalities to these proteins allowing deeper biological insight into the parasite's mechanisms of establishment and immune evasion (Lee *et al.,* 2019;

Dalton *et al.,* 2003). In the future these limitations could be minimised through use of fractionation of the global samples prior to mass spectrometry, separating the parasite tegument from the internal components, a technique that has previously been used in the investigation of *F. hepatica* showing an increase in number of proteins identified when compared to the soluble somatic fraction alone (Haçariz *et al.,* 2012). A further method of increasing the number of peptides resolved could be the use of anion and cation mixed bed ion exchange, which has been shown to increase peptide recovery and in turn increase the number of peptide hits returned follow LC-MS/MS analysis (Motoyama *et al.,* 2007).

6.2.2 Isolation and proteomic characterisation of C. daubneyi EVs

Investigation of adult *C. daubneyi* ES products confirmed the presence of EVs secreted during *in vitro* culture (Huson *et al.,* 2018). EVs were successfully purified utilising both DC and SEC methodologies, with TEM and proteomics combined with LC-MS/MS confirming their presence and allowing characterisation of exosome and microvesicle sub-populations through both investigation of size and also identification of exosomal and microvesicle markers utilising the ExoCarta database (Keerthikumar *et al.,* 2016). SEC purification methods produced an EV yield of higher purity with no observed instances of aggregation as with those from the DC method. With a lack of aggregation observed in the SEC purified samples and potential benefits for functional helminth EV analysis (Davis et al., 2019), SEC EVs were utilised in the downstream functional analysis of *C. daubneyi* EVs and their specific effects on microbial populations and for EV surface protein shaves. However, DC EV samples were utilised in the proteomic profiling in line with a number of alternative helminth EV studies (Marcilla *et al.,* 2012; Zhu *et al.,* 2016). At present all proteomic investigations of helminth EV populations have utilised DC as a purification method, with the

exception of one (Davis et al., 2019), and thus the use of EVs purified through DC allowed direct comparison of results to previous studies (Cwiklinski *et al.,* 2015; Nowacki *et al.,* 2015; Tzelos *et al.,* 2016). Whilst these two methodologies were utilised in this thesis, there are further purification techniques such as density gradient centrifugation, sucrose cushion centrifugation and immuno-affinity isolation that could also be tested in order to identify the best method for purification of *C. daubneyi* EVs generating the highest yield whilst maintaining functionality (Davis *et al.,* 2019). In order to fully investigate the functionality of EVs isolated using each purification method, EVs could be subject to treatment with host cells and then their proteomic profiles analysed against the host transcriptomes for each method as has been carried out for EVs from *O. viverrini* and *Typanosoma cruzi* (Chaiyadet *et al.,* 2015).

Chapter 4 successfully addressed a thesis aim through the resolution of the *C. daubneyi* EV proteome, elucidating a dataset that can be probed in order to identify key proteins that interact with host cells allowing successful immunomodulation of the host immune response. In addition, trypsin shaving of SEC purified EVs and their subsequent analysis using mass spectrometry elucidated the key surface proteins that could be utilised as potential biomarkers in the diagnosis of infections as well as those potentially involved in interaction with host cells allowing their successful uptake allowing them to exert their effects (Mathivanan *et al.,* 2010; Robbins & Morelli, 2014). EV surface proteins, especially those with demonstrated immunogenicity could be further investigated in the development of a diagnostic test allowing the diagnosis of paramphistomosis (Kip *et al.,* 2015). Further investigation would have to be carried out in order to identify the specific protein(s) of interest recognised in the blood of infected hosts and thus, not in uninfected animals, as well ensuring the animals being investigated had no other helminth infections that could be affecting the EVs or their

surface proteins in order to confirm their suitability as a diagnostic. Another important factor in utilising EV surface proteins as diagnostics for infection is the potential half-life of EVs within their host (Kip *et al.,* 2015). EV half-lives have been subject to investigation in humans yet are still to be examined in helminth infections.

C. daubneyi EVs were here shown to have antimicrobial effects with a reduction in microbial population growth in eight out of the ten species to which they were treated signifying the potential presence of antimicrobial peptides contained either within or on the surface of the EVs or a more generalised killing mechanism. Due to *C. daubneyi* residency within its final host the potential of microbial population alteration could be a key to understanding its mechanisms of survival as well as its negative effects on the host observed including weight loss and reduced milk yield (Fitzpatrick, 2013; Leung *et al.,* 2018). A further experiment investigating the effects of *C. daubneyi* derived EVs on specific ruminant microbial populations could elucidate the mechanisms through which establishment is successful with many bacterial species also capable of modulating the host immune response (Cattadori *et al.,* 2016).

When looking at potential biomarkers, the absence of cathepsins in both the soluble somatic and egg fractions was of particular interest following their identification in numerous helminth species and their characterised roles in parasite migration and activity in the suppression of the host immune response (Robinson *et al.,* 2008). However, this absence in the proteomic data is likely due to low level abundance as cathepsins have previously been described in *C. daubneyi* ES products (Huson, PhD Thesis) and here an abundance of cathepsins were also observed both within EVs themselves, as well as on the EV surface membrane, suggesting *C. daubneyi* EVs role in the successful establishment of infection through direct interaction with host cells

or tissues. It is also important to note that although parasitic helminth EVs have been shown to interact with recipient host cells, the mechanisms through which they interact with recipient host cells is still not resolved (Wang *et al.,* 2015; Zhu *et al.,* 2016).

6.2.3 Bioinformatic interrogation of C. daubneyi transcriptome for detoxification families

Advances in many aspects of molecular biology now allow the identification and characterisation of protein families and here has allowed the resolution of those potentially involved in detoxification in adult *C. daubneyi*. Previous studies into helminth detoxification ability have been hampered by lack of sequence data available. However, developments in these technologies now allows for a comprehensive analysis of helminth parasites through genomic and transcriptomic datasets becoming readily available to aid analysis. Production of these datasets allows identification of protein families involved in xenobiotic detoxification (Brophy et al. 2012).

The resolution of a *C. daubneyi* transcriptome allows an improved understanding of its fundamental biology as it contains thousands of genes that can be linked to global proteomic analyses allowing resolution of their proteomic profiles and subsequent characterisation. Transcriptomes have now been produced for a variety of helminth parasites allowing discovery experiments to be carried out elucidating key aspects of their functional biology (Choudhary *et al*., 2015; Santos *et al*., 2016; Young *et al*., 2011; Haçariz *et al.,* 2012). With no anthelmintic compounds currently licensed for use against *C. daubneyi* in the U.K and oxyclozanide remaining the recommended treatment option which is currently only available through off license prescription (Arias *et al.,* 2013; Rolfe & Boray, 1987) it is imperative that the fundamental biology of the parasite is resolved allowing identification of detoxification pathways present

within the parasite that can be considered in the development of new compounds in the future allowing the development of resistance to the compounds to be minimised.

Utilising a bioinformatics pipeline, this thesis investigated the presence of six known protein superfamilies involved in xenobiotic detoxification in the *C. daubneyi* transcriptome. The major Phase I detoxification families, cytochrome P450 (CYP), monoamine oxidase (MAO) and flavin-containing monooxygenase (FMO) were investigated with positive identification of sequences homologous to those of members of the CYP and MAO superfamilies identified. The major Phase II detoxification families glutathione transferase (GST), sulfotransferase (SULT) and UDP-Glycosyltransferase (UGTs), were also investigated identifying sequences homologous to GSTs and SULTs within the transcriptome. Interestingly, the only detoxification proteins identified through mining of the transcriptome to be identified in the resolved proteomes of the soluble somatic, egg and EV fractions were GSTs. However, this does not conclusively confirm the other detoxification families' absence, instead they may not appear in the proteomic analysis due to low expression levels as well as possibly requiring stimulation in order to be synthesised, with CYPs yet to be biochemically identified in any parasitic helminths (Precious & Barrett, 1989; Barrett, 1998). Many helminth studies have also noted an expansion of the GST superfamily (Huson *et al.*, 2018; Matouskova *et al.*, 2016). Of particular interest was the identification of Sigma class GST in EVs, which have only been previously described in the EVs released by human cancer cells, and a single helminth parasite - *F. hepatica*, where it has been suggested to have a predominant role in immunomodulation (Skog *et al.*, 2008; Cwiklinski *et al.*, 2015).

It is important to remember that the detoxification families identified in this study would need to be functionally validated through RNAi experimentation in order to confirm their role in detoxification of xenobiotic compounds. RNAi has been previously shown as a successful mechanism of suppression of specific proteins in parasitic helminths, allowing either their reduction or complete exclusion (Rindali *et al.,* 2008; McGonigle *et al.,* 2008), thus validating protein function as well as permitting testing of potential treatment compounds. However, RNAi investigations are still under ongoing development in helminths. To date there have been no experiments involving RNAi in rumen fluke and so methods would need to be developed and optimised prior to experimentation, however, as many helminth protocols have been optimised there are many methods that could be examined such as soaking or electroporation as utilised in *F. hepatica* (Rinaldi *et al.,* 2008). These developments of RNAi are vital in the further expansion of our understanding of the fundamental biology of *C. daubneyi,* especially in the validation of therapeutic targets.

A further area of research could be in the recent developments of CRISPR/Cas9 genome editing. Despite their complexity, studies have shown the ability to carry out genome editing on two parasitic flatworms, *S. mansoni* and *O. viverrini* (Arusan *et al.,* 2019; Ittiprasert *et al.,* 2019). Whilst RNAi allows the reduction in transcription of specific proteins it does not allow increased expression of specific groups and editing of their genomic DNA as in CRISPR. The potential of developing genetically modified parasites would allow elucidation of their mechanisms of establishment as well as potential mechanisms of control (McVeigh & Maule, 2019) that would be of great use in the study of *C. daubneyi* with its increasing prevalence and fractured understanding of its fundamental biology.

The aforementioned study of glycosylation could also be relevant when considering RNAi studies in *C. daubneyi*. Glycan inhibition has been suggested as a mechanism through which newly excysted juveniles (NEJs) migrate along the digestive tract in helminth species such as *F. hepatica*, representing an important role in the successful establishment of infection (Garcia-Campos *et al.*, 2017). It is important to note than in order for such experimentation to take place an *in vitro C. daubneyi* life-cycle would have to first be optimised of which attempts have so far been unsuccessful (Jones, PhD Thesis). With understanding of glycosylation's involvement in host-pathogen interactions could potentially lead to the development of treatment options.

6.3 SUMMARY

The research carried out throughout this thesis has addressed all of the aims set out. Paramphistomosis represents a significant cause of morbidity and mortality in livestock in tropical and sub-tropical regions (Sanabria & Romero, 2008). With *C. daubneyi* infections now recognised as widespread across the U.K and with APH veterinary investigations surveillance data (APH VIDA) suggesting an increased prevalence in recent years (Jones *et al.,* 2015, 2016) it is important to resolve aspects of the parasite's fundamental biology of which the current knowledge remains fractured due to paramphistomosis in Europe long regarded as clinically insignificant (Sargison *et al.,* 2016). This work represents the first proteomic investigation into *C. daubneyi* soluble somatic, egg and EV fractions that may prove useful in both comparative work with other helminth species as well as identifying potential vaccine candidates and diagnostic markers of infection that are likely to be needed in the future. EVs were purified for the first time from ES products and their antimicrobial properties confirmed. EV biogenesis pathways were also investigated at a transcript level and the presence of proteins capable of immune modulation within EVs highlights their biogenesis methods as a potential target for treating infection through inhibiting their successful establishment within the host. Transcript level studies also identified a range of detoxification families present in *C. daubneyi* that can be included in the future design of anthelmintic compounds should they be required. All data sets produced here can be utilised in future studies, with the combining of omic techniques allowing further understanding of these newly emerging parasites fundamental biology.

CHAPTER 7.

REFERENCES

REFERENCES

Aatonen, M. T., Ohman, T., Nyman, T. A., Laitinen, S., Gronholm, M., Siljander, P. RM. (2014). Isolation and characterisation of platelet-derived extracellular vesicles. *Journal of Extracellular Vesicles*. 3(1), 24692.

Abels, E. R., Breakefield, X. O. (2016). Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Molecular Neurobiology.* 36(3), 301-312.

Aebersold, R., Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*. 422, 198-207.

Aguayo, V., Valdes, B., Espino, A. M. (2018). Assessment of Fasciola hepatica glutathione S -transferase as an antigen for serodiagnosis of human chronic fascioliasis. *Acta Tropica.* 186, 41–49.

Akin, D. E., Rigsby, L. L. (1987). Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. *Applied and Environmental Microbiology*. 53, 1987-1995.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology.* 215, 403–10.

Alvarez, L. I., Solana, H. D., Mottier, M. L., Virkel, G. L., Fairweather, I. Lanusse, C. E. (2005). Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology.* 131, 501-510.

Alvinerie, M., Dupuy, J., Eeckhoutte, C., Sutra, J., Kerboeuf, D. (2001). In vitro metabolism of moxidectin in *Haemonchus contortus* adult stages. *Parasitology Research.* 87(9), 702-704.

Alzieu, J. P., Dorchies, P. (2007). Reemergence of cattle paramphistomiasis in France: Current review of epidemiology, pathophysiology and diagnosis. *Bulletin de l'Académie vétérinaire de France.* 160(2), 93-99.

Andaloussi, S. EL., Mager, I., Breakefield, X. O., Wood, M. J. A. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. *Nature Reviews: Drug Discovery.* 12, 347-357.

Anderson, L., Amaral, M. S., Beckedorff, F., Silva, L. F., Dazzani, B., Oliveira, K. C., Almeida, G. T., Gomes, M. R., Pires, D. S., Setubal, J. C., DeMarco, R., Verjovski, Almeida, S. (2015). Schistosoma mansoni Egg, Adult Male and Female Comparative Gene Expression Analysis and Identification of Novel Genes by RNA Seq. *PLoS Neglected Tropical Diseases*. 9(12).

Angelot, F., Seilles, E., Biichle, S., Berda, Y., Gaugler, B., Plumas, J., Dignat-George, F., Tiberghien, P., Saas, P., Garnache-Ottou, F. (2009). Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases. *Haematologica,* 94(11). 1502–1512.

Anuracpreeda, P., Wanichanon, C., Sobhon, P. (2008). *Paramphistomum cervi*: Antigenic profile of adults as recognized by infected cattle sera. *Experimental Parasitology*. 118, 203-207.

Arias, M. S. S., Sanchís, J., Francisco, I., Francisco, R., Piñeiro, P., Cazapal-Monteiro, C., Cortiñas, F. J. J., Suárez, J. L. L., Sánchez-Andrade, R., Paz-Silva, A., Sanchis, J., Francisco, I., Francisco, R., Pineiro, P., Cazapal-Monteiro, C., Cortinas, F. J., Suarez, J. L., Sanchez-Andrade, R., Paz-Silva, A. (2013). The efficacy of four anthelmintics against *Calicophoron daubneyi* in naturally infected dairy cattle. *Veterinary Parasitology*. 197, 126–129.

Arunsan, P., Ittiprasert, W., Smout, M. J., Cochran, C. J., Mann, V. H., Chaiyadet, S., Karinshak, S. E., Sripa, B., Young, N. D., Sotillo, J., Loukas, A., Brindley, P. J., Laha, T. (2019). Programmed knockout mutation of liver fluke granulin attenuates virulence of infection-induced hepatobiliary morbidity. *eLife.* 8, e41463.

Augot, D., Abrous, M., Rondelaud, D., Dreyfuss, G. (1996). *Paramphistomum daubneyi* and *Fasciola hepatica*: the redial burden and cercarial shedding in *Lymnaea*

truncatula submitted to successive unimiricidial cross-exposures. *Parasitology Research.* 82, 623-627.

Bae, Y. A., Ahn, E. G., Lee, S. H., Kim, S. H., Vai, G. B., Kang, I. (2013). Differential activation of diverse glutathione transferases of *Clonorchis sinensis* in response to the host bile and oxidative stressors. *PLoS Neglected Tropical Disease.* 7, e2211.

Bae, Y. A., Kim, J. G., Kong, Y. (2016). Phylogenetic characterization of *Clonorchis sinensis* proteins homologous to the sigma-class glutathione transferase and their differential expression profiles. *Molecular and Biochemical Parasitology.* 206(1-2), 46–55.

Baggerman, G., Vierstraete, E., De Loof, A., Schoofs, L. (2005). Gel-Based versus Gel-Free proteomics: A review. *Combinatorial Chemistry & High throughput screening.* 8, 669-677.

Ballweber, L.R. (2006). Diagnostic Methods for Parasitic Infections in Livestock. *Veterinary Clinics: Food Animal Practice*. 22(3), 695-705.

Barrett, J. (1997). Biochemical Pathways in Parasites. Analytical Parasitology. M. T. Rogan. Berlin, Heidelberg, 543, Springer Berlin Heidelberg. 1-31.

Barrett, J. (1998). Cytochrome P450 in parasitic protozoa and helminths. *Pharmacology, Toxicology and Endocrinology.* 121(1-3), 181-183.

Barrett, J. (2001). Comparative Glutathione S-Transferase (GSTs) Inhibition Assay in the Whole Extract of Fasciola hepatica and Sheep Liver Tissue by Hexachlorophene. *Iran J Public Health.* 30(3-4), 125-128.

Barrett, J. (2009). Forty years of helminth biochemistry. *Parasitology.* 136(12), 1633.

Barrett, J., Jefferies, J., Brophy, P. M. (2000). Parasite proteomics. *Parasitology Today.* 16(9), 400-403.

Baumann, E. (1976). Ueber sulfosauren im harn. *Dtsch. Chem. Ges.* 54-58.

Becker, A., Thakur, B. K., Weiss, J. M., Kim, H. S., Peinado, H., Lyden, D. (2016). Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell.* 30(6), 836-848.

Belanche, A., Oliver, G. F. (2012). Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. *Journal of Animal Science.* 90(11), 4802.

Bellet, C., Green, M, J., Vickers, M., Forbes, A., Berry, E., Kaler, J. (2016). Ostertagia spp., rumen fluke and liver fluke single- and poly-infections in cattle: An abattoir study of prevalence and production impacts in England and Wales. *Preventive Veterinary Medicine.* 132, 98-106.

Beltrao, P., Bork, P., Krogan, N. J., Noort, V. (2013). Evolution and functional cross-talk of protein post-translational modification. *Molecular Systems Biology.* 9, 714.

Benedetti, M. S. (2001). Biotransformation of xenobiotics by amine oxidases. *Fundamental and Clinical Pharmacology.* 15(2), 75-84.

Benedetti, M. S., & Tipton, K. F. (1998). Monoamine oxidases and related amine oxidases as phase I enzymes in the metabolism of xenobiotics. *Journal of Neural Transmission. Supplement.* 149–171.

Bergmann, S., Rohde, M., Hammerschmidt, S. (2004) Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pneumoniae is a surface-displayed plasminogen-binding protein. *Infection Immunology*. 72, 2416-2419.

Berriman, M., Lustigman, S., McCarter, J. P. (2007). Genomics and Emerging Drug Discovery Technologies. *Expert Opinion on Drug Discovery.* 2, supp1.

Board, P. G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L. S., Schulte, G. K. (2000). Identification, characterisation, and crystal structure of the omega class glutathione transferase. *Journal of Biological Chemistry.* 275(24), 798-806.

Booth, A. M., Fang, Y., Fallon, J. K., Yang, J.M., Hilderth, J. E., Gould, S. J. (2006). Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. Journal of Cellular Biology. 172(6), 923-935.

Brennan, G. P., Fairweather, I., Trudgett, A., Hoey, E., McCoy., McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., Brophy, P. M. (2007). Understanding Triclabendazole resistance. *Experimental and Molecular Pathology.* 82(22), 104-109.

Brindley, P. J., Mitreva, M., Ghedin, E., Lustigman, S. (2009). Helminth Genomics: The Implications for Human Health. *PLoS Neglected Tropical Diseases.* 3(1), e538.

Brophy, P.M., Mackintosh, N., Morphew, R.M., (2012). Anthelmintic metabolism in parasitic helminths: proteomic insights. *Parasitology* 139, 1205–1217.

Brosschot, T. P., Reynolds, L. A. (2018). The impact of a helminth-modified microbiome on host immunity. *Mucosalimmunology.*11, 1039-1046.

Buck, A. H., Coakley, G., Simbari, F., McSorley, H. J., Quintana, J. F., Le Bihan, T., Kumar, S., Abreu-Goodger, C., Lear, M., Harcus, Y., Ceroni, A., Babayan, S. A., Blaxter, M., Ivens, A., Maizels, R. M. (2014). Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nature Communications*. 5(1). 5488.

Buckley, D. B., Klaassen, C. D. (2009). Induction of Mouse UDP-Glucuronosyltransferase mRNA Expression in Liver and Intestine by Activators of Aryl-Hydrocarbon Receptor, Constitutive Androstane Receptor, Pregnane X Receptor, Peroxisome Proliferator-Activated Receptor α, and Nuclear Factor Erythroid 2-Related Factor 2. *Drug Metabolism and Disposition.* 37(4), 847-856.

Burmeister, C., Lüersen, K., Heinick, A., Hussein, A., Domagalski, M., Walter, R. D. (2008). Oxidative stress in *Caenorhabditis elegans*: protective effects of the omega class glutathione transferase (GSTO-1). *FASEB Journal.* 325, 25-37.

Caban-Fernandez, K., Gaudier, J. F., Espino, A. M. (2011). Characterisation and differential expression of a ferritin protein from *Fasciola hepatica. Molecular Biochemical Parasitology.* 182(1-2), 54-61.

Cancela, M., Acosta, D., Rinaldi, G., Silva, E., Duran, R., Roche, L., Zaha, A., Carmona, C., Tort, J. F. (2008). A distinct repertoire of cathepsins is expressed by juvenile invasive *Fasciola hepatica. Biochimie.* 90(10), 1461-1475.

Cao, X., Hong, Y., Zhang, M., Han, Y., Wu, M., Wang, X. (2014). Cloning, expression and characterization of protein disulfide isomerase of Schistosoma japonicum. *Experimental Parasitology*. 146C, 43-51.

Caspers, GJ., Leunissen, J. A. M., Jong, W. W. (1995). The expanding small heat-shock protein family, and structure predicitions of the conserved "a-crystallin domain". *Journal of Molecular Evolution.* 40, 238-248.

Cass, C. L., Johnson, J. R., Califf, L. L., Xu, T., Hernandez, H. J., Stadecker, M. J., Yates, J. R., Williams, D. L. (2007). Proteomic analysis of Schistosoma mansoni egg secretions. Molecular Biochemical Parasitology. 155(2), 84-93.

Castro, E., Sigrist, C.J., Gattiker, A., Bulliard, V., Petra, S., Langendijk-Genevaux, P.S., Gasteiger, E., Bairoch, A., Hulo, N., (2006). ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research*. 34, 362-365.

Cattadori, M., Sebastian, A., Hao, H., Katani, R., Albert, I., Eilertson, K. E., Kapur, V., Pathak, A., Mitchell, S. (2016). Impact of Helminth Infections and Nutritional Constraints on the Small Intestine Microbiota. *PLoS ONE.*

Chaiyadet, S., Sotillo, J., Smout, M., Cantacessi, C., Jones, M. K., Johnson, M. S., Loukas, A. (2015). Carcinogenic Liver Fluke Secretes Extracellular Vesicles That Promote Cholangiocytes to Adopt a Tumorigenic Phenotype. *The Journal of infectious diseases.* 212(10), 1636–1645.

Chargaff, E., West, R. (1946). The biological significance of the thromboplastic protein of blood. *Journal of Biological Chemistry*. 166(1), 189-197.

Charlier, J., Velde, F., van der Voort, M., Van Meensel, J., Lauwers, L., Cauberghe, V., Vercruysse, J. and Claerebout, E. (2019). ECONOHEALTH: Placing helminth infections of livestock in an economic and social context. 212(1-2), 62-67.

Chemale, G., Morphew, R., Moxon, J. V., Morassuti, L., LaCourse, J., Barrett, J., Johnston, D. A., Brophy, P. M. (2006). Proteomic analysis of glutathione transferases from the liver fluke parasite, *Fasciola hepatica. Proteomics.* 6, 6263-6273.

Chen, S., Liang, M., Chia, J. N., Ngsee, J. K., Ting, A. E. (2001). Rab8b and its interacting TRIP8b are involved in regulated secretion in AtT20 cells. *Journal of Biological Chemistry.* 276(16), 13209-13216.

Cheung, K. H., Keerthikumar, S., Roncaglia, P., Subramanian, S. L., Roth, M. E., Samuel, M., Anand, S., Gangoda, L., Gould, S., Alexander, R., Galas, D., Gerstein, M. B., Hill, A. F., Kitchen, R. R., Lotvall, J. (2016). Extending gene ontology in the context of extracellular RNA and vesicle communication. *Journal of Biomedical Semantics.* 7(19).

Choi, DS., Kim, DK., Kim, YK., Gho, Y. S. (2013). Proteomics, Transcriptomics and Lipidomics of exosomes and ectosomes. *Proteomics.* 13(10-11), 1554-1571.

Choudhary, V., Garg, S., Chouraisa, R., Hasnani, J. J., Patel, P. V., Shah, T. M., Bhatt, V. D., Mohapatra, A., Blake, D. P., Joshi, C. G. (2015). Transcriptome analysis of the adult rumen fluke Paramphistomum cervi following next generation sequencing. *Gene.* 570(1), 64-70.

Chryssafidis, A. L., Fu, Y., Waal, T. D., Mulcahy, G. (2015). Standardisation of egg-viability assays for Fasciola hepatica and Calicophoron daubneyi: A tool for evaluating new technologies of parasite control. *Veterinary Parasitology.* 210 (1-2),, 25-31.

Chuang, S., Velkov, T., Horne, J., Porter, C. J. H., Scanlon, M. J. (2008). Characterisation of the Drug Binding Specificity of Rat Liver Fatty Acid Binding Protein. *American Chemical Society.* 51(13), 3755-3764.

Cioli, D., Pica-Mattoccia, L., Basso, A., Guidi, A. (2014). Schistosomiasis control: praziquantel forever? *Molecular and Biochemical Parasitology.* 195(1), 23-29.

Clay, G. M., Sutterwala, F. S., Wilson, M. E. (2014). NLR proteins and parasitic disease. *Immunology Research*. (1-3), 142-152.

Coakley, G., Buck, A. H., Maizels, R. M. (2016). Host parasite communications-Messages from helminths for the immune system: Parasite communication and cell-cell interactions. *Molecular Biochemical Parasitology.* 208(1), 33-40.

Coakley, G., McCaskill, J., Borger, J., Simbari, F., Robertson, E., Millar, M., Harcus, Y., McSorley, H., Maizels, R. and Buck, A. (2019). Extracellular Vesicles from a Helminth Parasite Suppress Macrophage Activation and Constitute an Effective Vaccine for Protective Immunity. *Cell Reports.* 19(8), 1545-1557.

Cocucci, E., Racchetti, G., Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 19, 43–51.

Crescitelli, R., Lasser, C., Szabo, T. G., Kittel, A., Eldh, M., Dianzani, I., Buza, E. I., Lotvall, J. (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosome. *Journal of Extracellular Vesicles.* 2.

Cuesta-Astroz, Y., Oliveira, F. S., Nahum, L. A., Oliveira, G. (2017). Helminth

secretomes reflect different lifestyles and parasitized hosts. *International Journal of Parasitology.* 17, 30082-30088.

Curwen, R., Ashton, P., Johnston, D., Wilson, R. (2004). The Schistosoma mansoni soluble proteome: a comparison across four life-cycle stages. *Mol. Biochem. Parasitol.* 138, 57–66.

Cvilink, V., Lamka, J., Skalova, L. (2008). Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths. *Drug Metabolism Reviews*. 41(1), 8-26.

Cvjetkovic, A., Lotvall, J., Lasser, C. (2014). The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. *Journal of Extracellular Vesicles.* 3.

Cwiklinski, K., Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P. J., Brennan, G. P., O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J. P., Robinson, M. W. (2015). The Extracellular Vesicles of the Helminth Pathogen, *Fasciola hepatica*: Biogenesis Pathways and Cargo Molecules Involved in Parasite Pathogenesis. *Molecular and Cellular Proteomics.* 14(12), 3258-3273.

Dalton, J. P., Brindley, P. J., Knox, D. P., Brady, C. P., Hotez, P. J., Donnelly, S., O'Neill, S. M., Mulcahy, G., Loukas, A. (2003). Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. *International Journal for Parasitology.* 33, 621-640.

Das, A., Srinvasan, M., Ghosh, S., Mande, S. S. (2016). Xenobiotic metabolism and gut microbiomes. *PLoS ONE.* 11(10), e0163099.

Davis, C. N., Phillips, H., Tomes, J. J., Swain, M. T., Wilkinson, T. J., Brophy, P. M., Morphew, R. M. (2019). The importance of extracellular vesicle purification for downstream analysis: A comparison of differential centrifugation and size exclusion chromatography for helminth pathogens. *PLoS Neglected Tropical Diseases.* 13(2), e0007191.

DeMarco, R., Verjovski-Almeida, S. (2009). Schistosomes-proteomics studies for potential novel vaccines and drug targets. *Drug Discovery Today.* 14(9-10), 472-478.

Diaz, P., Lomba, C., Pedreira, J., Arias, M., Sanchez-Andrade, R., Suarez, J. L., Diez-Banos, P., Morrondo Paz-Silva, P.A. (2006). Analysis of the IgG antibody response against Paramphistomidae trematode in naturally infected cattle. *Veterinary Parasitology.* 140, 281-288.

Díaz, P., Pedreira, J., Sánchez-Andrade, R., Suarez J.L., Arias M.S., Francisco I., Fernandez G.,Diez-Banos, P., Morrondo, P., Paz-Silva, A. (2007). Risk periods of infection by Calicophoron daubneyi (Digenea:Paramphistomidae) in cattle from oceanic climate areas. *Parasitology research*. 101(2), 339-342.

Dieterich, C., Clifton, S. W., Schuster, L. N., Chinwalla, A., Delehaunty, K., Dinkelacker, I., Fulton, L., Fulton, R., Godfrey, J., Minx, P., Mitreva, M., Roeseler, W., Tian, H., Witte, H., Yang, S.-P., Wilson, R. K. and Sommer, R. J. (2008). The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. *Nature Genetics.* 40, 1193–1198.

Dijk, vD., Sargison, N. D., Kenyon, F., Skuce, P. J. (2010). Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. *Animal.* 4(3), 377-392.

Doenhoff, M. J. (1997). A role for granulomatous inflammation in the transmission of infectious disease: schistosomiasis and tuberculosis. Parasitology. 115, 113-125.

Donald, A. D. (1994). Parasites, animal production and sustainable development. *Veterinary Parasitology*. 54(1-3), 27-47.

Donnelly, S., O'Neill, S.M., Stack, C.M., Robinson, M.W., Turnbull, L., Whitchurch, C. and Dalton, J.P. (2010). Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. *J. Biol. Chem*. 285, 3383-3392

Doolan, D. L., Apte, S. H., Proietti, C. (2014). Genome-based vaccine design: the

promise for malaria and other infectious diseases. *International Journal for Parasitology.* 44(12), 901–913.

Dowling, D. J., Hamilton, C. M., Donnelly, S., La Course, J., Brophy, P. M., Dalton, J., O'Neill, S. M. (2010). Major secretory antigens of the helminth *Fasciola hepatica* active a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. *Infection and Immunity.* 78(2), 793-801.

Doyle, M. A., Gasser, R. B., Woodcroft, B. J., Hall, R. S., Ralph, S. A. (2010). Drug target prediction and prioritization: using orthology to predict essentiality in parasite genomes. *BMC Genomics.* 11(222).

Dreyfuss, G. (2015). The mud snail (*Galba truncatula)* Ecology, parasitism and control. *Lambert Academic Publishing, Saarbrucken, Germany.*

Eberle, R., Brattig, N., Trusch, M., Schlüter, H., Achukwi, M., Eisenbarth, A., Renz, A., Liebau, E., Perbandt, M. and Betzel, C. (2015). Isolation, identification and functional profile of excretory–secretory peptides from Onchocerca ochengi. 142, 156-166.

Eduardo, S. L. (1983). The taxonomy of the family Paramphistomidae Fischoder, 1901 with special reference of the morphology of species occurring in ruminants. III. Revision of the genus *Calicophoron* Nasmark, 1937. *Systematic Parasitology.* 5, 25-79.

Eichenberger, R. M., Sotillo, J., Loukas, A. (2018). Immunobiology of parasitic worm extracellular vesicles. *Immunology and Cell Biology*. 96(7).

Emmanuel, B., Laurence, C., Philippe, H., Jean-Philippe, V., Andrei, Z. (2012) Computational Systems Biology of Cancer. Chapman & Hall/CRC Mathematical & Computational Biology.

Esteves, A., Joseph, L., Paulino, M., Ehrlich, R. (1997). Remarks on the Phylogeny

and Structure of Fatty Acid Binding Proteins from Parasitic Plathelminths. *International Journal for Parasitology.* 27(9), 1013-1023.

F.A. Rojo-Vázquez, A. Meana, F. Valcárcel, M. Martínez-Valladares. (2012). Update on trematode infections in sheep. *Veterinary Parasitology.* 189 (1), 15-38.

Falany, C. N. (1997). Enzymology of human cytosolic sulfotransferases. *The FASEB Journal.* 11(4), 206-216.

Ferreras, M. C., González-Lanza, C., Pérez, V., Fuertes, M., Benavides, J., Mezo, M., González-Warleta, M., Giráldez, J., Martínez-Ibeas, A. M., Delgado, L., Fernández, M. and Manga-González, M, Y. (2014). Calicophoron daubneyi (Paramphistomidae) in slaughtered cattle in Castilla y León (Spain). *Veterinary Parasitology.* 199 (3–4), 268-271.

Figeuroa-Santiago, O., Espino, A. M. (2014). *Fasciola hepatica* fatty acid binding protein induces the alternative activation of human macrophages. *Infection Immunology.* 82, 5005-5012.

Fitzpatrick, J. L. (2013). Global food security: the impact of veterinary parasites and parasitologists. *Veterinary Parasitology.* 195, 233-248.

Fitzpatrick, J.M., Johnston, D.A., Williams, G.W., Williams, D.J., Freeman, T.C., Dunne, D.W., Hoffmann, K.F. (2005). An oligonucleotide microarray for transcriptome analysis of *Schistosoma mansoni* and its application/use to investigate gender-associated gene expression. *Mol. Biochem. Parasitol.* 141, 1–13.

Forbes, A. (2017). Grassland management and helminth control on livestock farms. *Livestock.* 22(2), 81-85.

Forbes, A. (2019). Strategic planning for parasite control on livestock farms. *Livestock.* 24(2).

Foster, A. P., Otter, A., O'sullivan, T., Cranwell, M. P., Twomey, D. F. (2008). Rumen Fluke (paramphistomosis) in British cattle. *The Veterinary Record.* 162(16), 528.

Foti, R. S., Tyndale, R. F., Garcia, K. L., Sweet, D. H., Nagar, S., Sharan, S., Rock, D. A. (2016). "Target-Site" Drug Metabolism and Transport. *Drug Metabolism and disposition: the biological fate of chemicals.* 43, 1156-1168.

Fox, J. E., Austin, C. D., Boyles, J. K., Steffen, P. K. (1990). Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane. *Journal of Cell Biology.* 111(2), 483.

Franchini, G. R., Porfido, J. L., Shimabukuro, M. I., Burusco, M. F. R., Belgamo, J. A., Smith, B. O., Kennedy, M. W., Corsico, B. (2015). The unusual lipid binding proteins of parasitic helminths and their potential roles in parasitism and as therapeutic targets. *PLEFA.* 93, 31-36.

Frova, C. (2006). Glutathione transferases in the genomics era: New insights and perspectives. *Biomolecular Engineering.* 23(4), 149-169.

Fuertes, M., Pérez, V., Benavides, J., González-Lanza, M.C., Mezo, M., González-Warleta, M., Giráldez, F.J., Fernández, M., Manga-González, M.Y. and Ferreras, M.C. (2015). Pathological changes in cattle naturally infected by Calicophoron daubneyi adult flukes. *Veterinary Parasitology.* 209, 188–196.

Fujita, K., Kume, H., Matsuzaki, K., Kawashima, A., Ujike, T., Nagahara, A., Uemura, M., Miyagawa, Y., Tomonaga, T., Nonomura, N. (2017). Proteomic analysis of urinary extracellular vesicles from high Gleason score prostate cancer. *Scientific Reports.* 7, 42961.

Furi, I., Momen-Heravi, F., Szabo, G. (2017). Extracellular vesicle isolation: present and future. *Annals of Translational Medicine.* 5(12), 263.

Gamage, N., Barnett, A., Hempel, N., Duggleby, R. G., Windmill, K. F., Martin, J. L., McManus, M. E. (2005). Human Sulfotransferases and Their Role in Chemical Metabolism. *Toxicological Sciences.* 90(1), 5-22.

Garcia-Campos, A., Baird, A. W., Mulcahy, G. (2017). Migration of *Fasciola hepatica* newly excysted juveniles is inhibited by high-mannose and oligomannose-type N-glycan-binding lectins. *Parasitology.* 144(13), 1708, 1717.

Gardiner, C., Di Vizio, D., Sahoo, S., Thery, C., Witwear, K. W., Wauben, M., Hill, A. F. (2016). Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *Journal of Extracellular Vesicles.* 5, 32945.

Garg, G., Bernal, D., Trelis, M., Forment, J., Ortiz, J., Valero, M. L., Pedrola, L., Martinez-Blanch, J., Esteban, J. G., Ranganathan, S., Toledo, R., Marcilla, A. (2013). The transcriptome of *Echinostoma caproni* adults: Further characterisation of the secretome and identification of new potential drug targets. *Journal of Proteomics.* 89, 202-214.

Garg, G., Ranganathan, S. (2012). Helminth secretome database (HSD): a collection of helminth excretory/secretory proteins predicted from expressed sequence tags (ESTs). *BMC Genomics.* 13(s8), 2283.

Geary, T.G., Conder, G.A., Bishop, B. (2004). The changing landscape of antiparasitic drug discovery for veterinary medicine. *Trends in Parasitology.* 20, 449–455.

Gézsi, A., Kovács, Á., Visnovitz, T. and Buzás, E. (2019). Systems biology approaches to investigating the roles of extracellular vesicles in human diseases. 51(33).

Ghedin, E., Wang, S., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., Allen, J.E., Delcher, A.L., Guiliano, D.B., Miranda-Saavedra, D., Angiuoli, S. V, Creasy, T., Amedeo, P.,

Haas, B., El-Sayed, N.M., Wortman, J.R., Feldblyum, T., Tallon, L., Schatz, M., Shumway, M., Koo, H., Salzberg, S.L., Schobel, S., Pertea, M., Pop, M., White, O., Barton, G.J., Carlow, C.K.S., Crawford, M.J., Daub, J., Dimmic, M.W., Estes, C.F., Foster, J.M., Ganatra, M., Gregory, W.F., Johnson, N.M., Jin, J., Komuniecki, R., Korf, I., Kumar, S., Laney, S., Li, B.- W., Li, W., Lindblom, T.H., Lustigman, S., Ma, D., Maina, C. V, Martin, D.M. a, McCarter, J.P., McReynolds, L., Mitreva, M., Nutman, T.B., Parkinson, J., Peregrín-Alvarez, J.M., Poole, C., Ren, Q., Saunders, L., Sluder, A.E., Smith, K., Stanke, M., Unnasch, T.R., Ware, J., Wei, A.D., Weil, G., Williams, D.J., Zhang, Y., Williams, S. a, Fraser-Liggett, C., Slatko, B., Blaxter, M.L., Scott, A.L., (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science*. 317, 1756–60.

Ginger, C. D., Fairbairn, D. (1966). Lipid metabolism in helminth parasites. I. The lipids of *Hymenolepis diminuta* (Cestoda). *Journal of Parasitology.* 52, 1086-1096.

Girardini, J., Amirante, A., Zemzoumi, K., Serra, E. (2002). Characterization of an omega-class glutathione S-transferase from *Schistosoma mansoni* with glutaredoxin-like dehydroascorbate reductase and thiol transferase activity. *European Journal of Biochemistry.* 269(55), 12-21.

Gobert, G. N., Jones, M. K. (2008). Discovering New Schistosome Drug Targets: The role of Transcriptomics. *Current Drug Targets.* 9, 922-930.

Gomez, S., Adalid-Peralta, L., Palafox-Fonseca, H., Cantu-Robles, V., Soberón, X., Sciutto, E., Fragoso, G., Bobes, R., Laclette, J., Yauner, L. and Ochoa-Leyva, A. (2015). Genome analysis of Excretory/Secretory proteins in *Taenia solium* reveals their Abundance of Antigenic Regions (AAR). *Nature Scientific Reports.* 5, 9683.

Gonzalez-Begne, M., Lu, B., Han, X., Hagen, F. K., Hand, A. R., Melvin, J. E., Yates, J. R. (2009). Proteomic Analysis of Human Parotid Gland Exosomes by Multidimensional Protein Identification Technology (MudPIT). *Journal of Proteome Research.* 8(3), 1304-1314.

Gordon, D. K., Roberts, L. C. P., Lean, N., Zadoks, R. N., Sargison, N. D., Skuce, P. J. (2013). Identification of the rumen fluke, *Calicophoron daubneyi*, in GB livestock: possible implications for liver fluke diagnosis. *Veterinary Parasitology.* 195(1-2), 65-71.

Gordon, D. K., Zadoks, R. N., Stevenson, H., Saringson, N. D., Skuce, P. J. (2012). On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with Fasciola hepatica. 187, 436-444.

Gordon, D., Zadoks, R., Skuce, P., Sarigson, N. (2012). Confirmation of triclabendazole resistance in liver fluke in the UK. *Veterinary record.* 171, 159-160.

Gordon, D.K., Roberts, L.C.P., Lean, N., Zadoks, R.N., Sargison, N.D. and Skuce, P.J. (2013). Identification of the rumen fluke, Calicophoron daubneyi, in GB livestock: possible implications for liver fluke diagnosis. *Veterinary Parasitology.* 195, 65 –71.

Gould, S. J. Raposo, G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. (2013). *Journal of Extracellular Vesicles.* 2.

Gourbal, B. E., Guillou, F., Mitta, G., Sibille, P., Thèron, A., Pointer, J. P., Coustau, C. (2008). Exretory-secretory products of larval *Fasciola hepatica* investigated using a two-dimensional proteomic approach. *Molecular Biochemical Parasitology.* 161, 63-66.

Greening, D. W., Xu, R., Gopal, S. K., Rai, A., Simpson, R. J. (2016). Proteomic insights into extracellular vesicle biology – defining exosomes and shed microvesicles. *Expert review of Proteomics.* 14(1), 69-95.

Grote, A., Caffery, C. R., Rebello, K. M., Smith, D., Dalton, J. P., Lustigman, S. (2018). Cysteine proteases during larval migration and development of helminths in their final hosts. *PLoS Tropical Diseases.*

Guengerich, F. P. (2005). Human Cytochrome P450 Enzymes, in: *Cytochrome P450: Structure, Mechanism, and Biochemsitry*. (Ortiz de Montellano PR ed), Kluwer Academic / Plenum, New York.

Guerreiro, E. M., Vestad, B., Steffensen, L. A., Aass, H. C. D., Saeed, M., Ovestebo, R., Costea, D. E., Galtung, H. K., Soland, T. M. (2018). Efficient extracellular vesicle isolation by combining cell media modifications, ultrafiltration, and size-exclusion chromatography. *PLoS ONE.* 13(9), e0204276.

Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y. & Aebersold, R. (2000). *Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc. Natl Acad. Sci.* 97, 9390–9395.

Habig, W. H., Pabst , M. J., Jakoby, W. B. (1974). Glutathione S-transferases. The first enzymatic step in 580 mercapturic acid formation. *Journal of Biological Chemistry.* 249(22), 7130-7139.

Haçariz, O., Akgün, M., Kavak, P., Yüksel, B., Saugiroglu, M. (2015). Comparative transcripome profiling approach to glean virulence and immunomodulation related genes of Fasciola hepatica. *BMC Genomics*. 16(1).

Haçariz, O., Sayers, G., Baykal, A. T. (2012). A proteomic approach to investigate the distribution and abundance of surface proteins during the chronic stage of natural liver fluke infection in cattle. *Journal of proteome research.* 11(7), 3592-3604.

Hall, T., (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*

Hasegawa, T., Konno, M., Baba, T., Sugeno, N., Kikuchi, A., Kobayashi, M., Takeda, A. (2011). The AAA-ATPase VPS4 Regulates Extracellular Secretion and Lysosomal Targeting of α-Synuclein. *PLoS ONE.* 6(12), e29460.

Hewitson, J. P., Grainger, J. R., Maizels, R. M. (2009). Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Molecular Biochemical Parasitology.* 167(1), 1-11.

Hewitson, J. P., Harcus, Y. M., Curwen, R. S., Dowle, A. A., Atmadja, A. K., Ashton, P. D., Wilson, A., Maizels, R. M. (2008). The secretome of the filarial parasite, *Brugia malayi:* Proteomic profile of adult excretory–secretory products. *Molecular and Biochemical Parasitology.* 160(1), 8–21.

Hiemstra, T. F., Charles, P. D., Gracia, T., Hester, S. S., Gatto, L., Al-Lamki, R., Floto, R. A., Su, Y., Skepper, J. N., Lilley, K. S., Karet Frankl, F. E. (2014). *Human Urinary Exosomes as Innate Immune Effectors. Journal of the American Society of Nephrology*. 25(9), 2017–2027.

Hillyer, G. V. (2005). *Fasciola* antigens as vaccines against fascioliasis and schistosomiasis. *Journal of Helminthology.* 79 (3), 241-247.

Hong, C. S., Funk, S., Muller, L., Boyiadzis, M., Whiteside, TL. (2016). Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. *Journal of Extracellular Vesicles*. 5, 29289.

Hoog, J. L., Lotvall, J. (2015). Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *Journal of Extracellular Vesicles.* 4, 2860.

Horak, I. G. (1971). Paramphistomiasis of Domestic Ruminants. *Advances in Parasitology.* 9, 33-72.

Hotez, P. J. (2009). One world health: neglected tropical diseases in a flat world. *PLoS Neglected Tropical Diseases.* 3, e405.

Hotez, P. J., Brindley, P. J., Bethoney, J. M., King, C. H., Pearce, E. J., Jacobson, J. (2009). Helminth infections: the great neglected tropical disease. *Journal of Clinical Investigation*. 118(4), 1311–1321.

Hristov, M., Erl, W., Linder, S., Weber, P. C. (2004). Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood*. 104, 2761–2766.

Hu, G., Gong, A.-Y., Roth, A. L., Huang, B. Q., Ward, H. D., Zhu, G., LaRusso, N.

F., Hanson, N. D., Chen, X.-M. (2013). Release of Luminal Exosomes Contributes to TLR4-Mediated Epithelial Antimicrobial Defense. *PLoS Pathogens*. 9(4), e1003261.

Huang, S. C., Freitas, T. C., Amiel, E., Everts, B., Pearce, E. L., Lok, J. B. (2012). Fatty acid oxidation is essential for egg production by the parasitic flatworm *Scistosoma mansoni*. *PLoS Pathogens.* 8, e1002996.

Hugel, B., Martínez, M. C., Kunzelmann, C., Freyssinet, J. (2005). Membrane Microparticles: Two Sides of the Coin. *Physiology*. 20(1), 22-27.

Huson, K. M., Morphew, R. M., Allen, N. R., Hegarty, M. J., Worgan, H. J., Girdwood, S. E., Jones, E. L., Phillips, H. C., Vickers, M., Swain, M., Smith, D., Kingston-Smith, A. H., Brophy, P. M. (2018). Polyomic tools for an emerging livestock parasite, the rumen fluke *Calicophoron daubneyi*; identifying shifts in rumen functionality. *Parasites and Vectors.* 11(1), 617.

Huson, K. M., Oliver, N. A. M., Robinson, M. W. (2017). Paramphistomosis of Ruminants: An Emerging Parasitic Disease in Europe. *Trends in Parasitology*. 33(11), 836-844.

Huson, K., Brophy, P., Morphew, R. and Mackintosh, N. (2015). Identification of the rumen fluke Calicophoron daubneyi infecting cattle in Wales. *Advances in Animal Biosciences*. 6, 183.

Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends in Immunology.* 28(10), 429-436.

Ittiprasert, W., Mann, VH., Karinshak, S. E., Coghlan, A., Rinaldi, G., Sankaranarayanan, G., Chaidee, A., Tanno, T., KumKhaek, C., Prangtaworn, P., Mentink-Kane, M. M., Cochran, C. J., Driguez, P., Holroyd, N., Tracey, A., Rodpai, R., Everts, B., Hokke, C. H., Hoffmann, K. F., Berriman, M., Brindley, P. J. (2019). Programmed genome editing of the omega-1 ribonuclease of the blood fluke, *Schistosoma mansoni*. *eLife.* 8, e41337.

Iyanagi, T. (2007). Molecular mechanism of Phase I and Phase II Drug-metabolizing enzymes: implications for detoxification. *International review of cytology.* 35-112.

James, C. E., Hudson, A. L., Davey, M. W. (2009). Drug resistance mechanisms in helminths: is it survival of the fittest?. *Trends in Parasitology.* 25(7), 328-335.

Jhee, S. S., Shiovitz, T., Crawford, A. W., Cutler, N. R. (2001). Pharmacokinetics and pharmacodynamics of the triptan antimigrane agents: a comparative review. *Clinical pharmacokinetics.* 40, 189-205.

Johnston M.J.G., Macdonald J.A., Mckay D.M. (2009). Parasitic helminths: a pharmacopeia of anti-inflammatory molecules. *Parasitology.* 136,125–147.

Jones, R. (2017). Epidemeology of Rumen Fluke (*Calicophoron daubneyi*) on Welsh Farms. *PhD Thesis, Aberystwyth University.*

Jones, R., Brophy, P., Mitchell, E., Williams, H. (2017). Rumen fluke (Calicophoron daubneyi) on Welsh farms: Prevalence, risk factors and observations on co-infection with Fasciola hepatica. *Parasitology.* 144(2), 237-247.

Jowsey, I. R., Thomson, A. M., Flanagan, J. U., Murdock, P. R., Moorre, G. B. T., Meyer, D. J. (2001). Mammalian class sigma glutathione-S-transferase: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D2 synthase. *Biochemical Journal.* 359, 507-516.

Kajugu, P. E., Hanna, R. E., Edgar, H. W., McMahon, C., Cooper, M., Gordon, A., Barley, J. P., Malone, F. E., Brennan, G. P., Fairweather, I. (2015). *Fasciola hepatica*: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. *Veterinary Parasitology.* 212, 181-187.

Kanaoka, Y., Fujimori, K., Kikuno, R., Sakaguchi, Y., Urade, Y., Hayaishi, O. (2000). Structure and chromosomal localization for human and mouse genes for

hematopoietic prostaglandin D synthase. *European Journal of Biochemistry.* 267, 3315-3322.

Kaplan, R. M. (2004). Drug resistance in nematodes of veterinary importance: a status report. *Trends in Parasitology.* 20(10), 477-481.

Keerthikumar, S., Chisanga, D., Ariyaratne, D., Saffar, H. A., Anand, S., Zhao, K., Samuel, M., Pathan, M., Jois, M., Chilamkurti, N., Gangoda, L., Mathivanan, S. (2016). ExoCarta: A web-based compendium of exosomal cargo. *Journal of Molecular Biology.* 428(4), 688-692.

Kelly, B. J. G. (1948). Paramphistomum cervi in cattle in Ireland. *Irish Veterinary Journal*. 2, 241-242.

Kerboeuf, D., Blackhall, W., Kaminsky, R., Samson-Himmelstjerna. (2003). P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *International Journal of Antimicrobial Agents*. 22(3), 332-346.

Kifle, D. W., Sotillo, J., Pearson, M. S., Loukas, A. (2017). Extracellular vesicles as a target for the development of anti-helminth vaccines. *Emerging Topics in Life Sciences.* 1(6), 659-665.

Kim, DK., Kang, B., Kim, O. Y., Choi, Ds., Lee, J., Kim, S. R., Go, G., Yoon, Y. J., Kim, J. H., Jang, S. C., Park, KS., Choi, EJ., Kim, K. P., Desiderio, D. M., Kim, YK., Lotvall, J., Hwang, D., Gho, Y. S. (2013). EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *Journal of Extracellular Vesicles*. 2(1), 20384.

Kim, J. G., Ahn, C. S., Sripa, B., Eom, K. S., Kang, I., Sohn, W. M., Nawa, Y., Kong, Y. (2019). Clonorchis sinensis omega-class glutathione transferases are reliable biomarkers for serodiagnosis of clonorchiasis and opisthorchiasis. *Clinical Microbiology and Infection.* 25(1).

Kip, A. E., Balasegaram, M., Beijnen, J. H., Schellens, J. H. M., De Vries, P. J. Dorloa, T. P. C. (2015). Systematic review of biomarkers to monitor therapeutic response in leishmaniasis. *Antimicrobial Agents and Chemotherapy*. 1–14.

Kostadinova, A., Pérez-del-Olmo, A. (2019). The systematics of the Trematoda. Digenetic Trematoda. *AEMB.* 1154, 21-42.

Krueger, S. K., Williams, D. E. (2005). Mammalian Flavin-containing monooxygenases: structure/function, genetic polymorphisms, and role in drug metabolism. Pharmacology and Therapeutics.106(3), 357-387.

Kucharzewska, P., Belting, M. (2012). Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress. *Journal of Extracellular vesicles*. 2(1), 20304.

Kulas, J., Schmidt, C., Rothe, M., Schunck, W.-H. and Menzel, R. (2008). Cytochrome P450-dependent metabolism of eicosapentaenoic acid in the nematode Caenorhabditis elegans. Archives of Biochemistry and Biophysics 472, 65–75.

Laing, S. T., Ivens, A., Laing, R., Ravikumar, S., Butler, V., Woods, D. J., Gilleard, J. S. (2010). Characterization of the xenobiotic response of *Caenorhabditis elegans* to the anthelmintic drug albendazole and the identification of novel drug glucoside metabolites. *Biochemical Journal.* 432 (3), 505-516.

Laing, T., Kikuchi, T., Cotton, J. A. (2013). The genome and transcriptome of *Haemonchus contortus,* a key model parasite for drug and vaccine discovery. *Genome Biology*. 14, R88.

Lamenza, P., Ortiz, P., Ceriani, C., Solana, H. (2012). Identification and characterization of phase I detoxification enzymes in isolates of *Fasciola hepatica* to triclabendazole susceptible and resistant. In: Proceedings of the 24th International Conference of the World Association for the Advancement of Veterinary Parasitology; Perth, Australia.

Lang, D., Kalgutkar, A. S. (2003). Non-P450 Mediated Oxidative Metabolism of Xenobiotics. *Drug Metabolising Enzymes.* 14, 483-539.

Lasser, C., Jang, S. C., Lotvall, J. (2018). Subpopulations of extracellular vesicles and their therapeutic potential. *Molecular Aspects of Medicine.* 60, 1-14.

Lee, H. Y., Kim, E. G., Jung, H. R., Jung, J. W., Kim, H. B., Cho, J. W., Kim, K. M., Yi, E. C. (2019). Refinements of LC-MS/MS Spectral Counting Statistics Improve Quantification of Low Abundance Proteins. *Scientific Reports.* 9(13653).

Lee, JS., Yong, TS. (2004). Epression and cross-species reactivity of fatty acid-binding proteins of *Clonorchis sinensis. Parasitology Research.* 93(5), 339-343.

Leung, J. M., Graham, A. L., Knowles, S. C. L. (2018). Parasite-microbiota interactions with the vertebrate gut: synthesis through an ecological lens. *Frontiers in Microbiology.* 9.

Li, R. W., Li, W., Sun, J., Yu, P., Baldwin, R. L., Urban, J. F (2016). The effect of helminth infection on the microbial composition and structure of the caprine abomasal microbiome. *Scientific Reports.* 6: 20606.

Li, S., Tighe, S.W., Nicolet, C.M., Grove, D., Levy, S., Farmerie, W., Viale, A., Wright, C., Schweitzer, P.A., Gao, Y., Kim, D., Boland, J., Hicks, B., Kim, R., Chhangawala, S., Jafari, N., Raghavachari, N., Gandara, J., Garcia Reyero, N., Hendrickson, C., Roberson, D., Rosenfeld, J., Smith, T., Underwood, J.G., Wang, M., Zumbo, P., Baldwin, D.A., Grills, G.S., Mason, C.E., (2014). Multiplatform assessment of transcriptome profiling using RNAseq in the ABRF next generation sequencing study. *Nat. Biotechnol*. 32, 915–925.

Li, Z., Clarke, A. J., Beveridge, T. J. (1996). A major autolysin of *Pseudomonas aeruginosa*: subcellular distribution, potential tole in cell growth and division and secretion in surface membrane vesicles. *Journal of Bacteriology.* 178, 2479-2488.

Li, ZW., Li, X., Yu, QY., Xiang, ZH., Kishino, H., Zhang, Z. (2009). The small heat shock protein (sHSP) genes in the silkworm, *Bombyx mori*, and comparative analysis with other insect sHSP genes. *BMC Evolutionary Biology.* 9(215).

Liebau, E., Höppner, J., Mühlmeister, M., Burmeister, C., Lüersen, K., Perbandt, M. (2008). The secretory omega-class glutathione transferase OvGST3 from the human pathogenic parasite *Onchocerca volvulus*. *FEBS Journal.* 275, 3438-3453.

Linares, R., Tan, S., Gounou, C., Arraud, N., Brisson, A. R. (2015). High-speed centrifugation induces aggregation of extracellular vesicles. *Journal of Extracellular Vesicles.* 4 (1), 29509.

Line, K., Isupov, M. N., LaCourse, E. J., Cutress, D. J., Morphew, R. M., Brophy, P. M., Littlechild, J. A. (2019). 'X-ray structure of *Fasciola hepatica* Sigma class glutathione transferase 1 reveals a disulphide bond to support stability in gastro-intestinal environment. *Scientific Reports.* 9(1), 902.

Liu, F., Hu, W., Cui, S.-J., Chi, M., Fang, C.-Y., Wang, Z.-Q., Yang, PY., Han, Z.-G. (2007). Insight into the host–parasite interplay by proteomic study of host proteins copurified with the human parasite *Schistosoma japonicum. Proteomics.* 7(3), 450–462.

Loukas, A., Gaze, S., Mulvenna, J. P., Gasser, R. B., Brindley, P. J., Doolan, D. L., Bethony, J. M., Jones, M. K., Gobert, G. N., Driguez, P., McManus, D. P., Hotez, P. J. (2011). Vaccinomics for the major blood feeding helminths of humans. *Omics.* 15, 567-577.

Lubec, G., Afjehi-Sadat, L. (2007). Limitations and pitfalls in protein identifications by mass spectrometry. *Chem. Rev*. 107, 3568–3584.

Lv, Z., Wu, Z., Zhang, L. Ji, P., Cai, Y., Luo, S., Wang, H., Li, H. (2016). Genome mining offers a new starting point for parasitology research. *Parasitology Research.* 114(2), 399-409.

Mage, C., Bourgne, H., Toullieu, JM., Rondelaud, D., Dreyfuss, G. (2002). Fasciola hepatica and Paramphistomum daubneyi: changes in prevalences of natural infections in cattle and in Lymnaea truncatula from central France over the past 12 years. *Veterinary Research*. 33(5), 439-447.

Maizels, R. M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M. D., Allen, J. E. (2004). Helminth parasites – masters of regulation. *Immunological Reviews.* 201(1).

Maizles, R. M., McSorley, H. J. (2016). Regulation of the host immune system by helminth parasites. *Journal of Allergy and Clinical Immunology.* 138(3), 666-675.

Malrait, K., Verschave, S., Skuce, P., Van Loo, H., Vercruysse, J. and Charlier, J. (2015). Novel insights into the pathogenic importance, diagnosis and treatment of the rumen fluke (Calicophoron daubneyi) in cattle. *Veterinary Parasitology*. 207 (1–2), 134-139.

Marcilla, A., Martin-Jaular, L., Trelis, M., de Menezes-Neto, A., Osuna, A., Bernal, D., Fernandez-Becerra, C., Almeida, I. C., Portillo, H. A. (2014). Extracellular vesicles in parasitic disease. *Journal of Extracellular Vesicles.* 3 (1).

Marcilla, A., Trelis, M., Cortes, A., Sotillo, J., Cantalapiedra, F., Minguez, M. T., Valero, M. L., Sanchez del Pino, M. M., Munoz-Antoli, C., Toledo, R., Bernal,D. (2012). Extracellular Vesicles from Parasitic Helminths Contain Specific Excretory/Secretory Proteins and Are Internalized in Intestinal Host Cells. *PLoS One.* 7.

Martinez-Ibeas, A. M., Munita, M. P., Lawlor, K., Sekiya, M., Mulchay, G. Sayers, R. (2016). Rumen fluke in Irish sheep: prevalence, risk factors and molecular identification of two paramphistome species. *BMC Veterinary Research.* 12, 143.

Mashburn-Warren, L. M., Whitely, M. (2006). Special Delivery: vesicle trafficking in prokaryotes. *Molecular Microbiology.* 61(4).

Mason, C., Stevenson, H., Cox, A., Dick, I. (2012). Disease associated with immature paramphistome infection in sheep. Veterinary Record. 170, 343-344.

Mathieu, M., Martin-Jaular, L., Lavieu, G., Thery, C. (2019). Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nature Cell Biology.* 21, 9-17.

Matoušková, P., Lecová, L., Laing, R., Dimunová, D., Vogel, H., Raisová Stuchlíková, L., Nguyen, L., Kellerová, P., Vokřál, I., Lamka, J., Szotáková, B., Várady, M. and Skálová, L. (2019). UDP-glycosyltransferase family in Haemonchus contortus: Phylogenetic analysis, constitutive expression, sex-differences and resistance-related differences. *International Journal for Parasitology: Drugs and Drug Resistance.* 8(3), 420-429.

Matouskova, P., Vokral, I., Lamka, J., Skalova, L. The role of Xenobiotics-Metabolizing Enzymes in Anthelmintic Deactivation and Resistance in Helminths. *Trends in Parasitology.* 32(6), 481-491.

McCarthy, J. S., Lustigman, S., Yang, GJ., Barakat, R. M., García, H. H., Sripa, B. (2012). A research agenda for helminth diseases of humans: diagnostics for control and elimina- tion programmes. *PLoS Neglegted Tropical Disease.* 6, e1601.

McGettigan, P. A. (2013). Transcriptomics in the RNA-seq era. *Current Opinion in Chemical Biology.* 17(1), 4–11.

McGonigle, L., Mousley, A., Marks, N. J., Brennan, G. P., Dalton, J. P. (2008). The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduced gut penetration. *International Journal of Parasitology.* 38(2), 149-155.

McKay, D. M. (2009). The therapeutic helminth?. *Trends in Parasitology.* 25(3), 109–114.

McManus, D. P., Bryant, C. (1986). Biochemistry and physiology of *Echinococcis* In: *The Biology of Echinococcus and Hydatid Disease* (Edited by Thompson R. C. A.), 114-136. George Allen & Unwin Ltd, London.

McNeilly, T, N., Mitchell, M. C., Corbishley, A., Nath, M., Simmonds, H., McAteer, S, P. (2014) Optimizing the Protection of Cattle against *Escherichia coli* O157:H7 Colonization through Immunization with Different Combinations of H7 Flagellin, Tir, Intimin-531 or EspA. *PLoS ONE.* 10(5), e0128391.

McVeigh, P., Cwiklinski, K., Garcia-Campos, A., Mulcahy, G., O'Neill, S. M., Maule, A. G., Dalton, J. P. (2018). *In silico* analyses of protein glycosylating genes in the helminth *Fasciola hepatica* (liver fluke) predict protein-linked glycan simplicity and reveal temporally-dynamic expression profiles. *Nature Scientific Reports.* 8, 11700.

McVeigh, P., Maule, A. G. (2019). Can CRISPR help in the fight against parasitic worms?. *eLIFE.* 8. e44382.

McVeigh, P., McCammick, E. M., McCusker, P., Morphew, R. M., Mousley, A., Abidi, A., Saifullah, K. M., Muthusamy, R., Gopalakrishnan, R., Spithill, T. W., Dalton, J. P., Brophy, P. M., Marks, N. J., Maule, A. G. (2014). RNAi dynamics in juvenile *Fasciola* spp. Liver flukes reveals the persistence of gene silencing *In Vitro*. *PLoS Neglected Tropical Diseases.* 8(9), e3185.

Meemon, K., Grams, R., Vichasri-Grams, S., Hofmann, A., Korge, G., Viyanant, V., Upatham, E. S., Habe, S., Sobhon, P. (2004). Molecular cloning and analysis of stage and tissue-specific expression of cathepsin B encoding genes from *Fasciola gigantica. Molecular and Biochemical Parasitology.* 136(1), 1-10.

Mekonnen, G. G., Pearson, M., Loukas, A., & Sotillo, J. (2018). Extracellular vesicles from parasitic helminths and their potential utility as vaccines. *Expert Review of Vaccines.* 17(3), 197–205.

Midha, A., Janek, K., Niewienda, A., Henklein, P., Guenther, S., Serra, D. O., Schlosser, J., Hengge, R., Hartmann, S. (2018). The Intestinal Roundworm Ascaris

suum Releases Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation. *Frontiers in Cellular and Infection Microbiology*. 8.

Millar, M., Colloff, A., Scholes, S., Bovine, H. (2012). Disease associated with immature paramphistome infection. *Veterinary Record*. 171, 509-511.

Mishra, P. K., Palma, M., Bleich, D., Loke, P., Gause, W. C. (2014). Systemic impact of intestinal helminth infections. *Mucosal Immunology.* 7, 753-762.

Mitreva, M., Zarlenga, D. S., McCarter, J. P., Jasmer, D. P. (2007). Parasitic nematodes-From genomes to control. *Veterinary Parasitology.* 148(1), 31-42.

Mitreva, M., Zarlenga, D., McCarter, J., Jasmer, D. (2007). Parasitic nematodes – from genomes to control. *Veterinary Parasitology.* 148, 31-42.

Mol, E. A., Goumans, MJ., Doevendans, P. A., Sluijter, J. P. G., Vader, P. (2017). Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation. *Nanomedicine.* 13(6), 2061-2065.

Montaner, S., Galiano, A., Trelis, M., Martin-Jaular, L., del Portillo, H. A., Bernal, D., Marcilla, A. (2014). The role of extracellular vesicles in modulating the host immune response during parasitic infections. *Frontiers in Immunology*. 5.

Mordvinov, V. A., Shilov, A. G., Pakharukova, M. Y. (2017). Anthelmintic activity of cytochrome P450 inhibitors miconazole and clotrimazole: in-vitro effect on the liver fluke *Opisthorchis felineus.* 50(1), 97-100.

Morozova, O., Hirst, M., Marra, M.A., (2009). Applications of new sequencing technologies for transcriptome analysis. *Annu. Rev. Genomics Hum. Genet*. 10, 135–151.

Morphew, R. M. (2007). Survival strategies of the liver fluke Fasciola hepatica: a proteomic investigation at the host–parasite interface. *PhD thesis*. University of Wales Aberystwyth.

Morphew, R. M., MacKintosh, N., Hart, E. H., Prescott, M., LaCourse, E. J., Brophy, P. M. (2014). *In vitro* biomarker discovery in the parasitic flatworm *Fasciola hepatica* for monitoring chemotherapeutic treatment. *EuPA Open Proteomics.* 85-99.

Motoyama, A., Xu, T., Ruse, C. I., Wohlschlegel, J. A., Yates, R. R. (2007). Anion and cation mixed-bed ion exchange for enhanced multidimensional seperations for peptides and phosphopeptides. *Analytical Chemistry.* 79(10), 3623-3634.

Moxon, J. V., LaCourse, E. J., Wright, H. A., Perally, S., Prescott, M. C., Gillard, J. L., Barrett, J., Hamilton, J. V., Brophy, P. M. (2010). Proteomic analysis of embryonic *Fasciola hepatica*: Characterization and antigenic potential of a developmentally regulated heat shock protein. *Veterinary Parasitology.* 169, 62-75.

Mulcahy, L. M., Pink, R, C., Carter, D. R. F. (2014). Routes and Mechanisms of extracellular vesicle uptake. *Journal of Extracellular Vesicles*. 3(1).

Mulvenna, J. , Sripa, B. , Brindley, P. J., Gorman, J. , Jones, M. K., Colgrave, M. L., Jones, A. , Nawaratna, S. , Laha, T. , Suttiprapa, S. , Smout, M. J. and Loukas, A. (2010), The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*. *Proteomics*. 10, 1063-1078.

Munguía, B., Michelena, M., Melian, E., Saldaña, J., Ures, X., Manta, E., & Domínguez, L. (2015). Development of novel valerolactam-benzimidazole hybrids anthelmintic derivatives: Diffusion and biotransformation studies in helminth parasites. *Experimental Parasitology.* 153, 75–80.

Munir, W. A., Barrett, J. (1989). The metabolism of xenobiotic compounds by *Hymenolepis diminuta* (Cestoda: Cyclophyllidea). *Parasitology.* 91(1), 145-156.

Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., D'Douza-Schorey, C. (2009). Microvesicles: mediators of extracellular communication during cancer progression. *Journal of Cell Science.* 123, 1603-1611.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., & D'Souza-Schorey, C. (2009). ARF6-Regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. *Current Biology*. 19(22), 1875–1885.

Murphy, T. M., Power, E. P., Sanchez-Miguel, C., Casey, M. J., Toolan, D. P. (2008). Paramphistomes in Irish cattle. *The Veterinary Record.* 162, 25.

Mutapi, F. (2012). Helminth parasite proteomics: from experimental models to human infections. *Parasitology.* 139(9), 1195–1204.

Ndao, M. (2009). Diagnosis of parasitic diseases: old and new approaches. *Interdiscip. Perspect. Infect. Dis.* 15.

Neil, G., D'Angelo, G., Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology.* 19, 213-228.

Nelson, D. R. (2009). The Cyrochrome P450 Homepage. *Human Genomics*. 4(1), 59-65.

Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotog, O., Nebert, D. W. (1993). The P450 Superfamily: Update on New Sequences, Gene Mapping, Accession Numbers, Early Trivial Names of Enzymes, and Nomenclature. *DNA and Cell Biology*. 12(1), 1–51.

Nowacki, F. C., Swain, M. T., Klychnikov, O. I., Niazi, U., Ivens, A., Quintana, J. F., Hensbergen, P. J., Hokke, C. H., Buck, A. H., Hoffman, K. F. (2015). Protein and

small non-coding RNA-enriched extracellular veiscles are released by the pathogenic blood fluke *Schistosoma mansoni. Journal of Extracellular Vesicles.* 4(1), 28665.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry.* 250, 4007-4021.

Ojopi, E. P., Oliveira, P. S., Nunes, D. N., Paquola, A., DeMarco, R., Gregório, S. P., Aires, K. A., Menck, C. FM., Leite, L. CC., Verjovski-Almedia, S., Dias-Neto, E. (2007*).* A quantitative view of the transcriptome of Schistosoma mansoni adult-worms using SAGE. *BMC Genomics*. 8(1), 186.

Oliveira, D. L., Freie-de-Lima, C. G., Nosanchuk, J. D., Casadevall, A., Rodrigues, M. L., Nimrichter, L. (2010). Extracellular Vesicles from *Cryptococcus neoformans* Modulate Macrophage Functions. *Infection and Immunity.* 78 (4) 1601-1609.

Oliveira, G. (2007). The Schistosoma mansoni transcriptome: An update. *Experimental Parasitology.* 117(3), 229–235.

Olson, P. D., Cribb, T. H., Tkach, V. V., Bray, R. A., & Littlewood, D. T. J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda)11Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AY222082–AY222285. *International Journal for Parasitology*. 33(7), 733–755.

Omari, A. A., Murry, D. J. (2007). Pharmacogenetics of the Cytochrome P450 Enzyme System: Review of Current Knowledge and Clinical Significance. *Journal of Pharmacy Practice*. 20(3), 206-218.

Ortiz de Montellano, P. R. (2005). Cytochrome P450: structure, mechanism, and biochemistry (3rd ed), Kluwer Academic, New York.

Ortiz de Montellano, P. R., De Voss, J. J. (2002). Oxidizing species in the mechanism of cytochrome P450. *Natural product reports.* 19, 477-493.

Ozdal, N., Gul, A., Ilhan, F., Deger, S. (2010). Prevalence of *Paramphistomum* infection in cattle and sheep in Van Province, Turkey. *Helminthologia.* 47, 20-24.

Pakharukova, M., Ershov, N., Vorontsova, E. V., Katokhin, A. (2012). Cytochrome P450 in fluke Opisthorchis felineus: Identification and characterization. *Molecular and Biochemical Parasitology.* 181(2), 190-194.

Pankao, V., Sirisriro, A., Grams, R., Vichasri-Grams, S., Meepool, A., Kangwanrangsan, N. (2006). Classification of the parenchymal cells in *Fasciola* gigantica based on ultrstructure and their expression of fatty acid binding proteins (FABPs). *Veterinary Parasitology.* 142, 281-292.

Paraud, C., Gaudin, C., Pors, I. and Chartier, C. (2009). Efficacy of oxyclozanide against the rumen fluke *Calicophoron daubneyi* in experimentally infected goats. *The Veterinary Journal.* 180 (2), 265-267.

Parkinson, A. (1996). Biotransformation of xenobiotics, Klassen, C. D. eds. *Cassarett and Doull's Toxicology: The Basic Science of Poisons* 5th edition. 113-186. McGraw-Hill New York.

Pavan, C. K., Syaama, N. S., Devi, V. P. (2014). Outbreak of immature paramphistomosis in Nellore Jodipi sheep. *Journal of Parasitic. Disease*. 4–6.

Pearce, E. J., MacDonald, A. S. (2002). The immunobiology of schistosomes. *Nature Reviews Immunology*. 2, 499-511.

Perry, B. D., Randolph, T. F. (1999). Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Veterinary Parasitology.* 84, 145–168.

Perry, B.D., Grace, D., Sones, K. (2011). Livestock and Global Change Special Feature: Current drivers and future directions of global livestock disease dynamics. *Proc. Natl. Acad. Sci*. 110, 20871–20877.

Petalcorin, M, I, R., Joshua, G. W., Agapow, PM., Dolphin, C. T. (2005). The *fmo* of *Caenorhabditits elegans* and *C. briggsae*: characterisation, gene expression and comparative genomic analysis. *Gene.* 346, 83-96.

Pica-Mattoccia, L., Carlini, D., Guidi, A., Cimica, V., Vigorosi, F., & Cioli, D. (2006). The schistosome enzyme that activates oxamniquine has the characteristics of a sulfotransferase. *Memórias Do Instituto Oswaldo Cruz*. 101(suppl. 1), 307–312.

Pillai, A., Ueno, S., Zhang, H., Lee, J. M. (2005). Cecropin P1and novel nematode cecropins: a bacteria-inducible antimicrobial peptide family in the nematode *Ascaris suum. Biochemical Journal.* 390(1), 207-214.

Pillers, A.W.N. (1922). Paramphistomum cervi from a cow in Cheshire. *Veterinary Journal*. 78, 292–293.

Pinto, H. A., Assis, J. C. A., Silva, B. C. M., Goncalves, N. Q., Melo, A. L. (2019). Zygocotyle lunata as a model for in vivo screening of anthelmintic activity against paramphistomes: Evaluation of efficacy of praziquantel, albendazole and closantel in experimentally infected mice. *Experimental Parasitology*.199, 74-79.

Pitarch, A., Sanchez, M., Nombela, C., Gil, C. (2002). Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. *Molecular cellular proteomics.* 1(12), 967-982.

Prasanphanich, N., Mickum, M., Heimburg-Molinaro, J., Cummings, R. (2013). Glycoconjugates in Host-Helminth Interactions. *Frontiers in Immunology.* 4, 240.

Precious, W. Y., Barrett, J. (1989). The possible absence of cytochrome P-450 linked xenobiotic metabolism in helminths. *Biochimica et Biophysica Acto (BBA).* 992(2), 215-222.

Raines, P. S., Barrett, J. (1988). *Hymenolepis diminuta:* Lack of sulphate activation and sulphotransferase activity. *Experimental Parasitology.* 65(2), 209–213.

Ramos, C. R., Spisni, A., Oyama, S., Sforca, M. L., Ramos, H. R., Vilar, M. M., Alves, A. C., Figueredo, R. C., Tendler, M., Zanchin, N. I., Pertinhez, T. A. (2009). Stability improvement of the fatty acid binding protein Sm14 from *S. mansoni* by Cys replacement: structural and functional characterization of a vaccine candidate. *Biochim Biophys Acta.* 4, 655-662.

Rangel-Ruiz, L. J., Albores-Brahms, S. T., Gamboa-Aguilar, J. (2003). Seasonal trends of *Paramphistomum cervi* in Tabasco, Mexico. *Veterinary Parasitology.* 116, 217-222.

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., Geuze, H. J. B lymphocytes secret antigen-presenting vesicles. *Journal of Experimental medicine.* 183(3), 1161.

Raposo, G., Stoorvogel, W. (2013). Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology.* 200(4), 373.

Reamtong, O. (2013). Mass Spectrometry-based Parasite Proteomics. *Journal of Tropical Medical Parasitology.* 36, 40-48.

Record, M. (2014). Intercellular communication by exosomes in placenta: A possible role in cell fusion?. *Placenta.* 35(5), 297-302.

Reynolds, L. A., Finlay, B. B., Maizels, R. M. (2015). Cohabitation in the Intestine: Interactions among Helminth Parasites, Bacterial Microbiota and Host Immunity. Th*e Journal of Immunology.* 195(9), 4059-4066.

Reynolds, L. A., Smith, K. A., Filbey, K. J., Harcus, Y., Hewitson, J. P., Redpath, S. A., Valdez, Y., Yebra, M. J., Finlay, B. B., Maizels, R. M. (2015). Commensal-pathogen interactions in the intestinal tract. *Gut Microbes.* 5(4), 522-533.

Riberio, P., Webb, R. A. (1984). The occurance, synthesis and metabolism of 5-hydroxytryptamine and 5-hydroxytryptophan in the cestode *Hymenolepis diminuta*: a

high performance liquid chromatographic study. *Comparative Biochemistry and physiology*. 79(1), 159-164.

Rindali, G., Morales, M. E., Cancela, M., Castillo, E., Brindley, P. J. (2008). Development of functional genomic tools in trematodes: RNA interference and luciferase reporter gene activity in *Fasciola hepatica. PLoS Neglected Tropical Disease*. 2(7), e260.

Riveau, G., Deplanque, D., Remoué, F., Schacht, A.M., Vodougnon, H., Capron, M., Thiry, M., Martial, J., Libersa, C., Capron, A. (2012). Safety and immunogenicity of rSh28GST antigen in humans: Phase 1 randomized clinical study of a vaccine candidate against urinary schistosomiasis. *PLoS Neglected Tropical Disease*. 6, 1–8.

Robbins, P. D., Morelli, A. E. (2014). Regulation of immune responses by extracellular vesicles. *Nature Reviews Immunology*. 195–208.

Robinson, M. W., & Connolly, B. (2005). Proteomic analysis of the excretory-secretory proteins of the *Trichinella spiralis* L1 larva, a nematode parasite of skeletal muscle. *Proteomics.* 5(17), 4525–4532.

Robinson, M. W., Dalton, J. P., Donnelly, S. (2008). Helminth pathogen cathepsins proteases: it's a family affair. *Trends in Biochemical Sciences.* 33(12), 601-608.

Robinson, M. W., Greig, R., Beattie, K. A., Lamont, D. J., & Connolly, B. (2007). Comparative analysis of the excretory–secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis. International Journal for Parasitology.* 37(2), 139–148.

Robinson, M. W., Hutchinson, A. T., Donnelly, S., Dalton, J. P. (2010). Worm secretory molecules are causing alarm. *Trends in Parasitology*. 26(8), 371-372.

Robinson, M. W., Menon, R., Donnelly, S. M., Dalton, J. P., Ranganathan, S. (2009). An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen Fasciola hepatica: proteins associated with invasion and infection of the mammalian hos. *Molecular & Cellular Proteomics*. 8, 1891–1907.

Robinson, M. W., Tort, J. F., Lowther, J., Donnelly, S. M., Wong, E., Xu, W., Stack, C. M., Padula, M., Herbert, B., Dalton, J. P. (2008). Proteomics and Phylogenetic Analysis of the Cathepsin L Protease Family of the Helminth Pathogen *Fasciola hepatica. Molecular & Cellular Proteomics.* 7(6), 1111–1123.

Robinson, M.W., Dalton, J.P. (2009). Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. 364, 2763-2776.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S. (2009). An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen Fasciola hepatica: proteins associated with invasion and infection of the mammalian host. *Molecular and Cellular Proteomics.* 8, 1891–1907.

Robledo, D., Ronza, P., Harrison, P.W., Losada, A.P., Bermúdez, R., Pardo, B.G., José., Redondo, M., Sitjà., Bobadilla, A., Quiroga, M.I., Martínez, P. (2014|). RNAseq analysis reveals significant transcriptome changes in turbot (*Scophthalmus maximus*) suffering severe enteromyxosis. *BMC Genomics.* 15, 1149.

Rogowska-Wrzesinska, A., Le Bihan, MC., Thaysen-Andersen, M., Roepstorff, P. (2013). 2D gels still have a niche in proteomics. Journal of Proteomics. 88, 4-13.

Rogowska-Wrzesinska, A., Le Bihan, MC., Thaysen-Andersen, M., Roepstorff, P. (2013). 2D gels still have a niche in proteomics. *Journal of Proteomics.* 88, 4-13.

Rojo-Vazquez, F. A., Meana, A., Valcarcel, F., Martinez-Valladares, M. (2012). Update on trematode infection in sheep. *Veterinary Parasitology.* 189(1), 15-38.

Rolfe, P.F., Boray, J.C., (1987). Chemotherapy of paramphistomosis in cattle. *Australian Veterinary Journal*. 64, 328–332.

Ronquist, G., Brody, I. (1985). The protasome: its secretion and function in man. *Reviews on Biomembranes.* 822(2), 203-218.

Ross, P., Huang, Y., Marchese, J., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A. and Pappin, D. (2019). Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Molecular and Cellular Proteomics.* 3(12), 1154-1169.

Rubartelli, A., Lotze, M. T. (2007). Inside, ourside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends in Immunology.* 28(10), 429-436.

Sabol, S. Z., Stella, H., Hamer, D. (1998). A functional polymorphism in the monoamine oxidase A gene promoter. *Human Genetics.* 103, 273-279.

Saghir, N., Conde, P. J., Brophy, P. M., Barrett, J. (2001). Biochemical characterisation of a hydrophobic ligand binding protein from the tapeworm *Hymenolepis diminuta. International Journal of Parasitology.* 31(7), 653-660.

Sahoo, S., Murugavel, S., Devi, I. K., Vedamurthy, G. V., Gupta, S. C., Singh, B. P. (2013). Glyceraldehyde-3-phosphate dehydrogenase of the parasitic nematode Haemonchus contortus binds to complement C3 and inhibits its activity. *Parasite Immunology*. 35, 457-67.

Samuel, M., Chisanga, D., Liem, M., Keerthikumar, S., Anand, S., Ang, C.-S., Adda, C. G., Versteegen, E., Jois, M., Mathivanan, S. (2017). Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth. *Scientific Reports, 7(1).*

Sanabria, R. E. F., Romero, J. R. (2008). Review and update of paramphistomosis. Helminthologia. 45, 64-68.

Sanabria, R., Moreno, L., Alvarez, L., Lanusse, C., Romero, J. (2014). Efficacy of oxyclozanide against adult Paramphistomum leydeni in naturally infected sheep. Veterinary Parasitology. 206(3-4), 277-281.

Sangster, N. (1996). Pharmacology of anthelmintic resistance. *Parasitology.* 113, S201-S216.

Santini-Oliveira, M., Coler, R.N., Parra, J., Veloso, V., Jayashankar, L., Pinto, P.M., Ciol, M.A., Bergquist, R., Reed, S.G., Tendler, M. (2016). Schistosomiasis vaccine candidate Sm14/GLA-SE: Phase 1 safety and immunogenicity clinical trial in healthy, male adults. *Vaccine.* 34, 586–594.

Santos, L. N., Silva, E. S., Santos, A. S., De Sá, P. H., Ramos, R. T., Silva, A., Pacheco, L. G. C. (2016). De novo assembly and characterization of the Trichuris trichiura adult worm transcriptome using Ion Torrent sequencing. *Acta Tropica, 159, 132–141.*

Sargison, N. D., Shahzad, K., Mazeri, S., Chaudhry, U. (2019). A high throughput deep amplicon sequencing method to show the emergence and spread of *Calicophoron daubneyi* rumen fluke infection in United Kingdom cattle herds. *Veterinary Parasitology.* 268, 9-15.

Saringson, N., Francis, E., Davison, C., Bronsvoort, B. M., Handel, I., Mazeri, S. (2016). Observations on the biology, epidemiology and economic relevance of rumen flukes (Paramphistomidae) in cattle kept in a temperate environment. *Veterinary Parasitology.* 219, 7-16.

Sarmah, P. C., Laha, R., Bhattacharjee, K., Goswami, A., Raquib, M., KAkati, P. (2014). The consumption of rumen flukes of cattle in India. *Southeast Asian Journal of Tropical Medicine*. 45, 26-30.

Savina, A., Fader, C. M., Damiani, M. T., Colombo, M. I. (2004). Rab11 Promotes Docking and fusion of Multivesicular Bodies in a Calcium-Dependant Manner. *Traffic.* 6(2), 131-143.

Scarcella, S., Lamenza, P., Virkel, G., Solana, H. (2012). Expression differential of microsomal and cytosolic glutathione-S-transferases in *Fasciola hepatica* resistant to triclabendazole. *Molecular and Biochemical Parasitology.* 181(1), 37-39.

Schorey, J. S., Cheng, Y., Singh, P. P., & Smith, V. L. (2014). Exosomes and other extracellular vesicles in host-pathogen interactions. *EMBO Reports.* 16(1), 24–43.

Schwartz, R., Ting, C. S., King, J. (2001). Whole Proteome pI Values correlate with subcellular localizations of proteins for organisms within the three domains of life. *Genome Research.* 11, 703-709.

Selkirk, M. E., Davis, R. E., Gounaris, K., Maizels, R. M. (2018). Special issue: molecular and cellular biology of helminth parasites XI. *International Journal of Parasitology.* 48(5), 319-320.

Sen-Hai, Y., Kenneth, M. E. (1994). Epidemiology and morbidity of food-borne intestinal trematode infections. *World Health Organisation.*

Sey, O. (1980). Revision of the amphistomes of European ruminants. *Parasitologia Hungarcia.* 13, 13-25.

Shen, B., Wu, N., Yang, J. M., Gould, S. J. (2011). Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J. Biol. Chem.* 286, 14383–14395.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O. (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proceedings of the National Academy of Sciences of the United States of America.* 93, 14440-14445.

Shih, J. C., Chen, K., Geha, R. M. (1998). Determination of regions important for monoamine oxidase (MAO) A and B substrate and inhibitor selectivities. *Journal of Neural Transmitters.* 52, 1-8.

Simons, M., Raposo, G. (2009). Exosomes – vesicular carriers for intercellular communication. *Current Opinions in Cell Biology*. 21, 575–81.

Simpson, R. J., Karla, H., Mathivanan, S. (2012). ExoCarta as a resource for exosomal research. *Journal of Extracellular Vesicles.* 1(1).

Singh, R. P., Sahai, B. N., Jha, G. J. (1984). Histopathology of the duodenum and rumen of goats during experimental infections with *Paramphistomum cervi*. *Veterinary Parasitology*. 15, 39-46.

Sirisina, S., Chawengkirttikul, R., Sermswan, R. (1991). Immunodiagnosis of opisthorchiasis. *Southeast Asian Journal of Tropical Medicine and Public Health*. 22, 179-183.

Sirisriro, A., Grams, R., Vichasri-Grams, S., Ardseungneon, P., Pankao, V., Meepool, A. (2002). Production and characterisation of a monoclonal antibody against recombinant fatty acid binding protein of *Fasciola gigantica. Veterinary Parasitology*. 105, 119-129.

Skálová, L., Cvilink, V., Bártíková, H., Lamka, J., Szotáková, B., 2010. Biotransformation of Xenobiotics in Lancet Fluke (Dicrocoelium dendriticum). In: LaMann, G. V. (Ed.), Veterinary Parasitology. NOVA Publishers, pp. 251–271.

Skuce, P. J., Morgan, E. R., Dijk van, J., Mitchell, M. (2013). Animal health aspects of adaptation to climate change: beating the heat and parasites in a warming Europe. *Animal.* 7(s2), 333-345.

Smythies, L. E., & Smythies, J. R. (2014). Exosomes in the gut. *Frontiers in Immunology.* 5, 104.

Sommer, A., Rickert, R., Fischer, P., Steinhart, H., Walter, R. D., Liebau, E. (2003). A Dominant Role for Extracellular Glutathione S-Transferase from *Onchocerca*

volvulus Is the Production of Prostaglandin D2. *Infections and Immunity.* 71(6), 3603-3606.

Sotillo, J., Doolan, D., & Loukas, A. (2016). Recent advances in proteomic applications for schistosomiasis research: potential clinical impact. *Expert Review of Proteomics.* 14(2), 171–183.

Sotillo, J., Pearson, M., Potriquet, J., Becker, L., Pickering, D., Mulvenna, J., Loukas, A. (2016). Extracellular vesicles secreted by *Schistosoma mansoni* contain protein vaccine candidates. *International Journal for Parasitology.* 46(1), 1-5.

Soulsby, J. L. (1965). Textbook of Veterinary Clinical Parasitology. Volume 1 – Helminths. *Blackwell Scientific Punlications Oxford, Oxford.*

Spence, S. A., Fraser, G. C., Chang, S. (1996). Response in milk production to the control of gastro-intestinal nematode and paramphistome parasites in dairy cattle. *Australian Vesterinary Journal*. 74, 456-459.

Sripa, J., Laha, T., Sripa, B. (2017). Characterization and functional analysis of fatty acid binding proteins from the carginogenic liver fluke, *Opisthorchis viverrini. Parasitology International.* 66(4), 419-425.

Stuart, R. (2016). Functional genomic approaches to understanding anthelmintic metabolism in the liver fluke *Fasciola hepatica. PhD Thesis, Aberystwyth University.*

Su, C., Su, L., Li, Y., Long, S. R., Chang, J., Zhang, W., Walker, W. A., Xavier, R. J., Cherayil, B. J., Shi, H. N. (2017). Helminth-induced alterations of the gut microbiota exacerbate bacterial colitis. *Mucosal Immunology.* 11, 144-157.

Subra, C., Grand, D., Laulagnier, K., Stella, A., Lambeau, G., Paillasse, M., Medina, P. D., Monsarrat, B., Perret, B., Silvente-Poirot, S., Poirot, M., Record, M. (2010). Exosome account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins. *Journal of Lipid Research.* 51, 2105-2120.

Svensson, K. J., Kucharzewska, P., Christianson, H. C., Sköld, C. S., Löfstedt, T., Johansson, M. C., Mörgelin, M., Bengzon, J., Ruf, W., Belting, M. (2011). Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signalling in endothelial cells. *PNAS.* 108(32), 13147-13152.

Szmidt-Adjidé, V., Abrous, M., Adjidé, C. C., Dreyfuss, G., Lecompte, A., Cabaret, J. and Rondelaud, D. (2000). Prevalence of Paramphistomum daubneyi infection in cattle in central France. Veterinary Parasitology. 87 (2–3), 133-138.

Tandon, V., Roy, B., Shylla, J. A., & Ghatani, S. (2014). Amphistomes. *Digenetic Trematodes*. 766, 365–392.

Taraboletti, G., D'Ascenzoy, S., Giusti, I., Marchetti, D., Borsotti, P., Millimaggi, D., Giavazzi, R., Pavan, A., Dolo, V. (2006). Bioavailability of VEGF in Tumor-Shed Vesicles Depends on Vesicle Burst Induced by Acidic pH. *Neoplasia.* 8(2), 96-103.

Taylor, A. B., Roberts, K. M., Cao, X., Clark, N. E., Holloway, S. P., Donati, E. Hart, P. J. (2017). Structural and enzymatic insights into species-specific resistance to schistosome parasite drug therapy. *Journal of Biological Chemistry.* 292(27), 11154–11164.

Taylor, D. D., Shah, S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. (2015). *Methods.* 87, 3-10.

Taylor, M. A. (2013). Parasite control in sheep: A risky business. *Small Ruminant Research*. 110, 88– 92.

Taylor, M. A., Coop, R. L., Wall, R. L. (2007). Veterinary Parasitology. John Wiley & Sons, Oxford, UK.

Teimoori, S., Arimatsu, Y., Laha, T., Kaewkes, S., Sereerak, P., Sripa, M., Tangkawattana, S., Brindley, P. J., Sripa, B. (2016). Chicken IgY-based coproantigen

capture ELISA for diagnosis of human opisthorchiasis. *Parasitology International.* 66(4), 443-447.

Tendler, M., Almeida, M., Simpson, A. (2015). Development of the Brazilian anti Schistosomiasis vaccine based on the recombinant fatty acid binding protein Sm14 plus GLA-SE adjuvant. *Frontiers in Immunology.* 6.

The Gene Ontology Consortium. (2017). Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Research.* 45(D1), D331-D338.

Thébaud, B., Stewart, D. J. (2012). Exosomes: cell garbage can, therapeutic carrier, or trojan horse? *Circulation.* 126, 2553-2555.

Thery, C., Ostrowski, M., Segura, E. (2009). Membrane vesicles as conveyors of immune responses. *Nature Reviews Immunology.* 9, 581-593.

Tilling, O. (2013). Rumen fluke in cattle in the UK: a review. *Livestock.* 18, 223-227.

Timanova-Atanasova, A., Jordanova, R., Radoslavov, G., Deevska, G., Bankov, I., Barrett, J. (2004). A native 13-kDa fatty acid binding protein from the liver fluke Fasciola hepatica. *Biochimica Et Biophysica Acta-General Subjects*. 1674(2), 200–204.

Timar, C. I., Lorincz, A. M., Csepanyi-Komi, R., Valyi-Nagy, A., Nagy, G., Buzas, E. I., Ivanyi, Z., Kittel, A., Powell, D. W., McLeish, K. R., Ligeti, E. (2012). Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood*, 121(3), 510–518.

Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S., Dijl, J. M. (2000). Signal Peptide-Dependent Protein Transport in *Bacillus subtilis*: a Genome-Based Survey of the Secretome. *Microbiology and Molecular Biology Reviews.* 64(3), 515-547.

Toet, H., Piedrafieta, D. M., Spithill, T. W. (2014). Liver fluke vaccines in ruminants: stratergies, progress and future opportunities. *International Journal for Parasitology.* 44(12), 915-927.

Toledo, R., Bernal, M. D., Marcilla, A. (2011). Proteomics of foodborne trematodes. *Journal of Proteomics.* 74, 1485-1503.

Toledo, R., Fried, B. (2017). Trematoda (flukes). *Emerging Topics in Life Science.* 1(6), 651–657.

Toolan D. P., Mitchell, G., Searle, K., Sheehan, M., Skuce, P. J. and Zadoks, R. N. (2015). Bovine and ovine rumen fluke in Ireland—Prevalence, risk factors and species identity based on passive veterinary surveillance and abattoir findings. *Veterinary Parasitology*. 212(3–4), 168-174.

Torre-Escudero, E., Gerlach, J. Q., Bennett, A. P. S., Cwiklinski, K., Jewhurst, H. L., Huson, K. M., Joshi, L., Kilcoyne, M., O'Neill, S., Dalton, J. P., Robinson, M. W. (2019). Surface molecules of extracellular vesicles secreted by the helminth pathogen Fasciola hepatica direct their internalisation by host cells. *PLOS Neglected Tropical Diseases.* 13(1).

Torre-Escudero, E., Robinson, M. W. (2017). Extracellular vesicle-mediated communication in host-parasite interactions: insight from *Fasciola hepatica. Annals of Translational Medicine.* 5(suppl 1), S8.

Torres-Rivera, A., Landa, A. (2008). Glutathione transferases from parasites: A biochemical view. *Acta Tropica.* 105(2), 99-112.

Torro-Escudero, E., Bennett, A. P. S., Clarke, A., Brennan, G. P., Robinson, M. W. (2016). Extracellular Vesicle Biogenesis in Helminths: more than one route to the surface?. *Trends in Parasitology.*

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science*. 319, 1244–1247

Tsai, I., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K., Tracey, A., Bobes, R., Fragoso, G., Sciutto, E., Aslett, M., Beasley, H., Bennett,

H., Cai, J., Camicia, F., Clark, R., Cucher, M., De Silva, N., Day, T., Deplazes, P., Estrada, K., Fernández, C., Holland, P., Hou, J., Hu, S., Huckvale, T., Hung, S., Kamenetzky, L., Keane, J., Kiss, F., Koziol, U., Lambert, O., Liu, K., Luo, X., Luo, Y., Macchiaroli, N., Nichol, S., Paps, J., Parkinson, J., Pouchkina-Stantcheva, N., Riddiford, N., Rosenzvit, M., Salinas, G., Wasmuth, J., Zamanian, M., Zheng, Y., Cai, X., Soberón, X., Olson, P., Laclette, J., Brehm, K. and Berriman, M. (2019). The genomes of four tapeworm species reveal adaptations to parasitism. *Nature.* 496, 57-63.

Twu, O., Johnson, P. J. (2014). Parasite extracellular vesicles: mediators of intracellular communications. *PLoS Pathogens.* 10.

Tzelos, T., Matthews, J. B., Buck, A. H., Simbari, F., Frew, D., Inglis, N. F., McLean, K., Nisbet, A. J., Whitelaw, C. B. A., Knox, D. P., McNeilly, T. (2016). A preliminary proteomic characterisation of extracellular vesicles released by the ovine parasitic nematode, *Teladorsagia circumcincta. Veterinary Parasitology.* 221, 84-92.

Umasuthan, N., Kasthuri, S. R., Lee, Y., Whang, I. (2012). A Novel Molluscan Sigma-Like Glutathione S-Transferase from Manila Clam, Ruditapes Philippinarum: Cloning, Characterization and Transcriptional Profiling. *Comparative Biochemsitry and Physiology.* 155(4), 539-550.

Umasuthan, N., Saranya Revathy, K., Lee, Y., Whang, I., Choi, C. and Lee, J. (2019). A novel molluscan sigma-like glutathione S-transferase from Manila clam, Ruditapes philippinarum: Cloning, characterization and transcriptional profiling. 155(4), 539-550.

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology.* 9, 654-659.

Valentim, C. L. L., Cioli, D., Chevalier, F. D., Cao, X., Taylor, A. B., Holloway, S. P., Pica-Marroccia, L., Guidi, A., Basso, A., Tsai, I. J., Berriman, M., Carvalho-Queiroz, C., Almeida, M., Aguilar, H., Frantz, D. E., Hart, P. J., LoVerde, P. T.,

Anderson, T. J. C. (2013). Genetic and Molecular Basis of Drug Resistance and Species-Specific Drug Action in Schistosome Parasites. *Science,* 342(6164), 1385–1389.

Van Niel, G., D'Angelo, G., Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature Reviews: Molecular Cell Biology.* 19, 213-228.

Van Niel, G., Porto-Carreiro, I., Simoes, S., Raposo, G. (2006). Exosomes: a common pathway for a specialized function. *Journal of Biochemistry.* 140, 13-21.

Veermas, R. E., Akpinar, G. G., Eldh, M., Gabrielsson, S. (2019). Immune Cell-Derived Extracellular Vesicles – Functions and Therapeutic Applications. *Trends in Molecular Medicine.* 25(5), 382-394.

Vokrál, I., Bártíková, H., Prchal, L., Stuchlíková, L., Skálová, L., Szotáková, B., Lamka, J., Várady, M., Kubíček, V. (2019). The metabolism of flubendazole and the activities of selected biotransformation enzymes in Haemonchus contortus strains susceptible and resistant to anthelmintics. 139(10), 1309-1316.

Vokral, I., Jirasko, R., Stuchlikova, L., Bartikova, H., Szotakova, B., Lamka, J., Varady, M., Skalova, L. (2013). Biotransformation of albendazole and activities of selected detoxification enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Veterinary Parasitology.* 196(3-4), 373-381.

Voss, T., Haberl, P. (2000). Observations on the reproducibility and matching efficiency of two-dimensional electrophoresis gels: consequences for comprehensive data analysis. *Electrophoresis.* 21, 3345-3350.

Waine, G. J. B. M., Yang, W., Kalinna, B., McManus, D. P. (1993). Cloning, molecular characterization, and functional activity of Schistosoma japonicum glyceraldehyde-3-phosphate dehydrogenase, a putative vaccine candidate against schistosomiasis japonica. *Infection Immunology*. 61, 4716-4723.

Walk, S. T., Blum, A. M., Ewing, S. AS., Weinstock, J. V., Young, V. B. (2010).

Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polgyrus. Inflammatory Bowel Disease.* 16(11), 1841-1849.

Waller, P. J. (1999). International approaches to the concept of intergrated control of nematode parasites of livestock. *International Journal for Parasitology.* 29(1), 155-164.

Waller, P. J. (2003). Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. *Animal Health Research Reviews*. 4(1), 35-44.

Wang, J., Kaletsky, R., Silva, M., Williams, A., Haas, L., Androwski, R., Landis, J., Patrick, C., Rashid, A., Santiago-Martinez, D., Gravato-Nobre, M., Hodgkin, J., Hall, D., Murphy, C. and Barr, M. (2019). Cell-Specific Transcriptional Profiling of Ciliated Sensory Neurons Reveals Regulators of Behavior and Extracellular Vesicle Biogenesis. *Current Biology*. 25(24), 3232-3238.

Wang, J., Zhao, F., Yu, C. X., Xiao, D., Song, L. J., Yin, X. R. (2013). Identification of proteins inducing short-lived antibody responses from excreted/secretory products of Schistosoma japonicum adult worms by immunoproteomic analysis. *Journal of Proteomics.* 87, 53-67.

Wang, L., Li, Z., Shen, J., Liu, Z., Liang, J., Wu, X., Sun, X., Wu, Z. (2015). Exosome-like vesicles derived by Schistosoma japonicum adult worms mediates M1 type immune- activity of macrophage. *Parasitology Research.* 114(5), 1865–1873.

Wang, Z., Martin, J., Abubucker, S., Yin, Y., Gasser, R.B., Mitreva, M. (2009). Systematic analysis of insertions and deletions specific to nematode proteins and their proposed functional and evolutionary relevance. *BMC Evolutionary Biology*. 9, 23.

Wastling, J. M., Armstrong, S. D., Krishna, R., Xia, D. (2012). Parasites, proteomes and systems: has Descartes' clock run out?. *Parasitology.* 139(9), 1103-1118.

Weinstock, J. V., Elliott, D. E. (2009). Helminths and the IBD hygiene hypothesis. *Inflammatory Bowel Disease.* 15, 128-133.

Wiklander, O. P. B., Brennan, M. A., Lotvall, J., Breakfield, X. O., Andaloussi, S. EL. (2019). Advances in therapeutic applications of extracellular vesicles. *Science Translational Medicine.* 11(492), eaav8521.

Willmott, S. (1950). On the species of *Paramphistomum* Fischoeder 1901 occuring in Britain and Ireland, with notes on some material from the Netherlands and France. *Journal of Helminthology.* 24, 155-170.

Willms, E., Cabanas, C., Mager, I., Wood, M. J. A., Vader, P. (2018). Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. *Frontiers in Immunology.* Review Article.

Wolstenholme, A. J., Fairweather, I., Prichard, R., Samson-Himmelstjerna, G., Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends in Parasitology.* 20(10), 469-476.

Wongratanacheewin, S., Pumidonming, W., Sermswan, R. W., Pipitgool, V., Maleewong, W. (2002). Detection of Opisthorchis viverrini in human stool specimens by PCR. *Journal of Clinical Microbiology.* 40, 3879-3880.

Wu, X.J., Sabat, G., Brown, J.F., Zhang, M., Taft, A., Peterson, N., Harms, A., Yoshino, T.P. (2009). Proteomic analysis of Schistosoma mansoni proteins released during in vitro miracidium-to-sporocyst transformation. *Mololecular Biochemical. Parasitol*ogy. 164, 32–44.

Xu, C., Li, C. YT., Kong, AN. T. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Archives of Pharmacalogical Research.* 28(249).

Yanez-Mo, M., Siljander, P. R. M., Andreu, Z., Zavec, A. B., Borrass, F. E. *et al.* (2015). Biological protperties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles.* 4(1), 27066.

Yang, Y., Cheng, J., Singhal, S., Saini, M., Pandya, U., Awasthi, S., Awasthi, Y. (2019). Role of GlutathioneS-Transferases in Protection against Lipid Peroxidation. 276, 19220-19230.

Youdim, M. B. H., Edmondson, D., Tipton, K. F. (2006). The therapeutic potential of monoamine oxidase inhibitors. *Nature Reviews Neuroscience.* 7, 295-309.

Young, N. D., Campbell, B. E., Hall, R. S., Jex, A. R., Cantacessi, C., Laha, T., Sohn, WM., Sripa, B., Loukas, A., Brindley, P. J., Gasser, R. B. (2010a). Unlocking the Transcriptomes of Two Carcinogenic Parasites, *Clonorchis cinensis* and *Opisthorchis viverrini*. *PLOS Neglected Tropical Diseases.* 4(6), e719.

Young, N.D., Hall, R.S., Jex, A.R., Cantacessi, C., Gasser, R.B., (2010b). Elucidating the transcriptome of *Fasciola hepatica* - A key to fundamental and biotechnological discoveries for a neglected parasite. *Biotechnology. Advances*. 28, 222– 231.

Zaiss, M. M., Harris, N. L. (2016). Interactions between the intestinal microbiome and helminth parasites. *Parasite Immunology.* 38(1), 5-11.

Zakeri, A., Hansen, E. P., Andersen, S. D., Williams, A. R., Nejsum, P. (2018). Immunomodulation by Helminths: Intracellular Pathways and Extracellular Vesicles. *Frontiers in Immunology.* 9, 2349.

Zamanian, M., Fraser, L. M., Agbedanu, P. N., Harischandra, H., Moorhead, A. R., Day, T. A., Bartholomay, L. C., Kimber, M. J. (2015). Release of Small RNA-containing Exosome-like Vesicles from the Human Filarial Parasite *Brugia malayi. PLOS Neglected Tropical Diseases.* 9(9), e0004069.

Zeeberg, B. R., Feng, W., Wang, G., Wang, M. D., Fojo, A. T., Sunshine, M., Narasimhan, S., Kane, D. W., Reinhold, W. C., Lababidi, S., Bussey, K. J., Riss, J.,

Barrett, J. C., Weinstein, J. N. (2003). GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biology.* 4(R28).

Zhao, J., Luo, R., Xu, X., Zou, Y., Zhang, Q., & Pan, W. (2015). High-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) reveals Argonaute-associated microRNAs and targets in *Schistosoma japonicum. Parasites & Vectors.* 8(1), 589.

Zhou, G., Stevenson, M.M., Geary, T.G., Xia, J., (2016). Comprehensive Transcriptome Metaanalysis to Characterize Host Immune Responses in Helminth Infections. *PLoS Neglected Tropical Disease.* 10(4), e0004624.

Ziniel, P.D., Karumudi, B., Barnard, A.H., Fisher, E.M.S., Thatcher, G.R.J., Podust, L.M., Williams, D.L., (2015). The Schistosoma mansoni Cytochrome P450 (CYP3050A1) Is Essential for Worm Survival and Egg Development. *PLoS Neglected Tropical Disease*. 9, 1–21.

Zintl A, García-Campos A, Trudgett A, Chryssafidis A, Talavera-Arce S, Fu Y, Egan S, Lawlor A, Negredo C, Brennan G, Hanna R, De Waal T, Mulcahy G. (2014). Bovine paramphistomes in Ireland. *Veterinary Parasitology*. 204(3-4),1 99–208.