Elucidating the developmental biology of *Haemonchus contortus* and other nematodes using a multi-omics approach

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DECLARATION

The work described in the thesis was performed in the Department of Veterinary Biosciences, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences of The University of Melbourne between 1 July 2016 and 30 June 2019. The scientific work was performed solely by the author, with the exception of the assistance which has been specifically acknowledged. The thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices. No part of this thesis has been submitted for any other degree or diploma.

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DISSEMINATION OF RESEARCH FINDINGS

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ABBREVIATIONS

cDNA: complementary DNA

CDS: coding DNA sequence

cGMP: cyclic guanosine monophosphate

CPM: counts per million

CRISPR: clustered regularly interspaced short palindromic repeats

CSF: cerebrospinal fluid

DA: dafachronic acid

daf: dauer formation associated gene

DAF-9: a cytochrome P450 encoded by *daf-9* gene

DAF-12: a nuclear hormone receptor encoded by *daf-12* gene

DDA: data-dependent acquisition

DIA: data-independent acquisition

ES: excretory/secretory

FA: fatty acid

FDR: false discovery rate

FPKM: fragments per kilobase per million mapped reads

GL: glycerolipids

GP: glycerophospholipids

GO: gene ontology

GPCR: G protein-coupled receptor

HCA: hierarchical cluster analysis

IGF-1: insulin-like growth factor 1

INS: insulin-like peptide coded by *ins* gene

ITS-1: first internal transcribed spacer

ITS-2: second internal transcribed spacer

KEGG: Kyoto Encyclopaedia of Genes and Genomes

LB: Luria Bertani medium

LBD: ligand-binding domain

LC-MS/MS: liquid chromatograph-mass spectrometry/mass spectrometry

LFQ: label-free quantification

LPE: lysophosphatidylethanolamine

LPC: lysophosphatidylcholine

MG: monoradylglycerol

mRNA: messenger RNA

miRNA: microRNA

NaClO: sodium hypochlorite

NR: non-redundant

ORF: open reading frame

PC: glycerophosphocholine

PCA: principal component analysis

PCR: polymerase chain reaction

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PK: polyketide

PR: prenol lipid

PPAR: peroxisome proliferator-activated receptor

RMSD: root-mean-square deviation

rRNA: ribosomal RNA

SCP/TAPS: sperm-coating protein/tpx/antigen 5/pathogenesis related-1/sc7

SP: sphingolipid

ST: sterol lipid

SR: saccharolipid

SRBC: serpentine receptor, class BC

SRG: serpentine receptor, class G

TCEP: tris (2-carboxyethyl) phosphine

TG: triradylglycerol

TGF-β: transforming growth factor β

TALEN: transcriptional activator-like effector nuclease

TCA: tricarboxylic acid

TFA: trifluoroacetic acid

ZFN: zinc-finger nuclease

SUMMARY

An *appraisal of current literature* (Chapter 1) revealed that many parasitic worms are pathogens of animals, causing major diseases and socioeconomic losses worldwide. Efforts to control roundworms (nematodes) are often compromised by widespread resistance to currently used treatments. Thus, there is a clear need to work toward new interventions, preferably based on a deep understanding of the molecular biology of nematodes and/or the relationship that they have with their host animals.

The *predominant focus* of the present thesis was on exploring aspects of the developmental biology of parasitic nematodes using advanced molecular ('omic) and bioinformatic technologies, with an emphasis on the barber's pole worm (*Haemonchus contortus*) - one of the most economically important parasites of ruminant livestock. The *specific aims* were: (1) to establish molecular data sets (resources) for *H. contortus*; (2) to explore RNA transcription and protein expression profiles in the developmental transition from free-living to parasitic larvae of *H. contortus* under well-defined conditions in vitro; (3) to construct dauer-like signalling pathways in *H. contortus* and some other nematodes (ascaridoids); and (4) to elucidate dauer-like signalling and the involvement of bile acid-like dafachronic acids during nematode development. All of these aims were achieved.

Addressing aim 1, comprehensive molecular (transcriptomic, proteomic and lipidomic) resources were established for *H. contortus* using advanced nucleic acid sequencing or mass spectrometry techniques (Chapters 2-4).

Addressing aim 2, these resources were utilised for in-depth explorations of molecular changes during the developmental switch from the free-living to the parasitic stage of *H. contortus* (Chapter 5). This work revealed extensive alterations in transcription and protein expression. There was a discordance between the mRNA transcription and protein expression changes, which appeared to relate to microRNA regulation at the post-transcriptional level and genes involved in signal transduction and signalling molecule interactions. Comparative studies with *C. elegans* indicated that molecules and canonical dauer signalling pathways integrate environmental cues and developmental processes in *H. contortus*.

Addressing aim 3, gene homologues involved in the dauer signalling pathways were inferred for *H. contortus* using genomic, transcriptomic and proteomic data sets (Chapter 6). Transcriptomic and proteomic studies of such homologues indicated similar gene transcription and protein phosphorylation profiles between the infective stage of *H. contortus* and the dauer stage of *Caenorhabditis elegans*. Although reduced sets of genes encoding G protein-coupled receptors, insulin-like peptides and cholesterol transporters were identified in *H. contortus*, similar functional roles of the signalling pathways were proposed for parasitic and free-living nematodes.

Addressing aim 4, the "dauer hypothesis" was tested in *H. contortus* using an integrated 'omics approach (Chapter 7). The dauer-like signalling pathways were shown to be activated during the developmental transition from the free-living to the parasitic stages of *H. contortus*, and were linked to an amplification of a 3-keto bile acid-like steroid hormone (i.e., dafachronic acid). This hormone bound to a nuclear receptor DAF-12 in vitro, and exhibited stimulatory effects on the larval activation and development of this parasite. These stimulatory effects appeared to be associated with a modulation of the dauer-like signalling cascades and lipid (glycerolipid and glycerophospholipid) metabolism. Specific chemical inhibition of dafachronic acid biosynthesis disrupted lipid metabolism and compromised larval activation and development, suggesting key roles for the hormone signalling module in the development of *H. contortus*. This work "opened the door" to exploring homologous signalling pathways in biologically distinct (ascaridoid) nematodes, including *Toxocara canis* (causing toxocariasis) and *Ascaris suum* (causing acariasis) (Chapters 8 and 9).

In conclusion, this thesis showed that an integrated use of these resources allows detailed explorations of signalling molecules, molecular processes and pathways likely associated with nematode development, adaptation and parasitism, and provides opportunities to identify novel intervention targets (Chapter 10). Although this work was focused mainly on *H. contortus* and two ascaridoid species, the multi-omics approach established herein could be readily used to explore a wide range of interesting and socioeconomically significant parasitic worms (including also trematodes and cestodes) at the molecular level, and to elucidate host-parasite interactions and disease processes.

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Chapter 1. Literature review

1.1. Introduction

Multicellular parasites, including trematodes, cestodes and nematodes, are significant pathogens of humans and animals, causing substantial disease burdens worldwide. For example, it has been estimated that more than one billion people are infected by parasitic nematodes (e.g., common roundworm, hookworms and whipworm), equating to ~5 million disability-adjusted life years (DALYs), which is comparable to the burden of tuberculosis, malaria, Ebola and Zika combined (Bethony et al., 2006; Hotez et al., 2009; Lo et al., 2017; Jourdan et al., 2018). In addition, nematodes affect livestock animals globally, causing substantial production losses estimated at tens of billion dollars annually (e.g., Fitzpatrick, 2013; Roeber et al., 2013).

The administration of anthelmintics has contributed to controlling some diseases caused by parasitic nematodes (McKellar and Jackson, 2004; Hotez, 2018). However, the excessive use of nematocides has led to the emergence and spread of drug resistance, particularly in nematodes of veterinary significance (Geerts and Gryseels, 2001; Kaplan, 2004; Wolstenholme et al., 2004; Sutherland and Leathwick, 2011; Playford et al., 2014). With this drug resistance issue and without effective vaccines against the vast majority of nematodes, it has become challenging to control nematodiases of humans and other animals. There is a clear need to work toward new interventions (drugs or vaccines), based on a profound understanding of the biology of parasitic nematodes at molecular level. The use of advanced technologies and tools could enable such a research focus.

In the past two decades, advances in technologies such as nucleic acid sequencing, mass spectrometry and computational biology (Aebersold and Mann, 2003; Domon and Aebersold, 2006; Metzker, 2010; Cantacessi et al., 2012; Goodwin et al., 2016) have accelerated many fields of biology. The sequencing of the genome of *Caenorhabditis elegans* (cf. *Caenorhabditis elegans* Sequencing Consortium, 1998) marked a turning point in the molecular exploration of metazoan organisms. The recent use of genomic, transcriptomic, proteomic and metabolomic tools has led to a profound understanding of areas including physiology, genetics, neurology and biochemistry in the ‘elegant worm’ (Girard et al., 2007; Corsi et al., 2015; van Assche et al., 2015; Howe et al., 2017; Watts

and Ristow, 2017), and also in other metazoans more generally (e.g., humans and helminths; Bürglin et al., 1998; Geary and Thompson, 2001; Markaki and Tavernarakis, 2010; Burns et al., 2015; Salinas and Risi, 2018). However, much less is understood about parasitic nematodes of major socioeconomic importance, such as *Trichuris trichiura* (clade I), *Ascaris suum* and *Brugia malayi* (clade III), *Strongyloides stercoralis* (clade IV) and *Haemonchus contortus* (clade V) (Stoltzfus et al., 2017; International Helminth Genomes Consortium, 2019; Jex et al., 2019). Fortunately, some of these parasitic nematodes are relatively closely related to the free-living nematode *Caenorhabditis elegans*, providing scope for comparative molecular investigations, such as in the area of developmental biology (e.g., Bento et al., 2010; Salinas and Risi, 2018). This is particularly true for parasitic nematodes of clade V, such as *H. contortus* - one of the economically most important parasites of livestock animals worldwide.

The main aims of this literature review were: (i) to provide a background on the development of nematodes, with a particular emphasis on the molecular aspects and developmental processes in *Caenorhabditis elegans*; (ii) to critically appraise the current state of knowledge of the developmental biology of parasitic nematodes and identify key knowledge gaps; (iii) to review salient aspects of *H. contortus*, with a focus on the recent advances in genomics, transcriptomic and proteomics as well as in vitro culturing systems; (iv) to make conclusions from this review to formulate the research aims of this thesis, to be tackled using advanced molecular and informatic tools.

1.2. Developmental biology of nematodes

Nematodes develop from an embryonated egg to an adult worm via multiple larval transitions. The developmental transition from one stage to another is reflected in a series of physical and physiological changes, such as moulting, feeding and metabolism (Bird, 1987; Lee, 2002; Rougvie and Moss, 2013). In response to environmental parameters, nematodes change their physical and physiological status (Rogers and Sommerville, 1957; Riddle and Albert, 1997; van Voorhies and Ward, 1999; Lützelschwab et al., 2005), leading to developmental variants, such as the dauer form in the free-living nematode *Caenorhabditis elegans* (see Cassada and Russell, 1975) and arrested development (hypobiosis) in some parasitic nematodes (e.g., *H. contortus* and *Toxocara canis*) (see

Michel, 1974; Gibbs, 1986; Nikolaou and Gasser, 2006; Schnieder et al., 2011; Fig. 1.1). In the past decades, there has been an improvement in our understanding of the developmental processes in nematodes through advances achieved in *Caenorhabditis elegans* areas, including signal transduction, biological pathways and gene regulation (e.g., Hu, 2007; Fielenbach and Antebi, 2008; Faunes and Larraín, 2016).

1.2.1. Signalling molecules involved in developmental regulation

In response to environmental conditions, pheromones (ascarosides), cyclic guanosine monophosphate (cGMP), transforming growth factor β (TGF-β), insulin/insulin-like peptides (INS) and hormones (dafachronic acids) serve as important signalling molecules for the developmental regulation in *Caenorhabditis elegans* (Fig. 1.2).

Ascarosides are small-molecule pheromones (glycosides) secreted into the environment, first discovered in the human intestinal parasite *Ascaris lumbricoides* (see Flury, 1912). It was found that these pheromones are synthesised in *Caenorhabditis elegans* in response to unfavourable conditions (e.g., high population density) (see Golden and Riddle, 1982, 1984). Since that time, 150 specific ascarosides have been identified in this free-living nematode (von Reuss et al., 2012), and linked to developmental regulation and behaviour (Ludewig and Schroeder, 2013). Such molecules appear to be produced by a broad range of free-living and parasitic nematodes (Choe et al., 2012). Particularly well-characterised ascarosides are ascr#2, #3 and #5, which are now known as ligands for G protein-coupled receptors (GPCR; DAF-37 and -38; SRBC-64 and -66; and SRG-36 and -37) and play roles in the dauer formation of *Caenorhabditis elegans* (see Ludewig and Schroeder, 2013; Butcher, 2017). This ascaroside and G protein signalling system links to the synthesis of secondary messengers, including cGMP, TGF-β and INS (Ludewig and Schroeder, 2013; Fig. 1.2).

Cyclic guanosine monophosphate (cGMP) acts downstream of G protein signalling in chemosensory neurons in *Caenorhabditis elegans* (see Bargmann, 2006). This second messenger depolarises cells by activating cGMP-gated ion channels, and likely plays a role in chemosensory transduction (Bargmann, 2006). Reduction in cGMP synthesis has been linked to dauer formation, and can be rescued by a cGMP analogue, suggesting a

key role in the developmental processes in this free-living nematode (Birnby et al., 2000; Hu, 2007).

Transforming growth factor β (TGF-β) members are intercellular signalling molecules recognised to regulate a range of cellular processes. In *Caenorhabditis elegans*, such molecules are encoded by five genes (*dbl-1*, *daf-7*, *unc-129*, *tig-2* and *tig-3*), but only one product (i.e., DAF-7 - a type of TGF-β) encoded by *daf-7* appears to be involved in the regulation of dauer in *Caenorhabditis elegans* (see Gumienny and Savage-Dunn, 2013). DAF-7 is expressed predominantly in a chemosensory (ASI amphid) neuron, and binds to its membrane receptors DAF-1 and DAF-4 of endocrine cells, assuming a role in the recovery from, or the bypassing of dauer (Hu, 2007; Gumienny and Savage-Dunn, 2013). Gene homologues coding for DAF-7 and its receptors have been identified in several parasitic nematodes, including *Ancylostoma caninum*, *Brugia malayi*, *Heligmosomoides polygyrus* (see Gomez-Escobar et al., 1997, 2000; Freitas and Arasu, 2005; McSorley et al., 2010). Some evidence also indicates that a TGF-β homologue and its receptors are involved in the activation of infective larvae of *Ancylostoma caninum* (canine hookworm; see Arasu, 2001) and *H. contortus* (barber's pole worm; see He et al., 2018).

Insulin-like peptides (INS) are secreted proteins orthologous to human insulin, encoded by 40 genes (*daf-28* and *ins-1* to *ins-39*) in *Caenorhabditis elegans* (see Pierce et al., 2001; Murphy and Hu, 2013). Although there is only one receptor, DAF-2, for these INS, they appear to have agonistic and antagonistic roles in the larval development of *Caenorhabditis elegans*. Previous studies have elucidated that DAF-28, INS-4 and INS-6 act as agonists of insulin/IGF-1 signalling and promote continuous larval development, whereas INS-1, INS-17 and INS-18 function as antagonists, promoting dauer formation in this free-living nematode (Hu, 2007; Murphy and Hu, 2013). Recent studies (Matsungaga et al., 2016; Zheng et al., 2018; Kaplan et al., 2019) reveal the intricate roles of INS in the developmental processes of *Caenorhabditis elegans*, which depend on the polarity of secretion of INS and are inferred to be regulated by positive and negative feedback mechanisms.

Hormones produced by cytochrome P450s also appear to play critical roles in regulating dauer formation in *Caenorhabditis elegans* (see Gerisch and Antebi, 2004; Held et al., 2006; Rottiers et al., 2006). Fractionation and characterisation of lipid extracts from wild-type *Caenorhabditis elegans* identified two distinct 3-keto-cholestenoic acid

metabolites of DAF-9/cytochrome P450 [(25S)-Δ4-dafachronic acid and (25S)-Δ7-dafachronic acid], which bind to DAF-12 and prevent dauer (see Motola et al., 2006). A recent comparative metabolomic analysis confirmed that (25S)-Δ7-dafachronic acid is the endogenous ligand for DAF-12 in *Caenorhabditis elegans* (see Mahanti et al., 2014). Interestingly, current evidence shows that the activation of DAF-12 occurs downstream of the cGMP, TGF-β and insulin/IGF-1 signalling - a "developmental checkpoint" in this free-living nematode (Lee and Schroeder, 2012; Schaedel et al., 2012; Antebi, 2013), although pathway composition and function may vary between nematodes (Stoltzfus et al., 2012, 2014).

Collectively, this information indicates that particular signalling molecules (ascarosides, cGMP, TGF-β, INS and dafachronic acids) are ligands for membrane or nuclear receptors, and serve as agonist(s) or antagonist(s) for the transduction of environmental cues through sensory neurons to endocrine tissues. Functional information for the signalling molecules and their receptors infers key intercellular and/or intracellular signalling cascade(s) and pathways, which are intimately involved in the regulation of development in *Caenorhabditis elegans* (Fig. 1.2).

1.2.2. Molecular pathways that integrate environmental signals and developmental plasticity

Detailed studies have revealed an involvement of cGMP, TGF-β, insulin/IGF-1 and dafachronic acid signalling cascades in the phenotypic plasticity of *Caenorhabditis elegans*, particularly in developmental variations in response to environmental conditions (Fielenbach and Antebi, 2008; Bento et al., 2010; Sommer and Ogawa, 2011; Lee and Schroeder, 2012; Butcher, 2017). Based on current knowledge, the framework for this development-associated signalling cascades is likely to be:

Environmental signals (e.g., ascarosides and food cues) are transduced via sensory neurons through cGMP signalling, then integrated into the parallel TGF-β and insulin/IGF-1 signalling to trigger steroid hormone biosynthesis, which controls molecular "decisions" on continuous development or dauer formation in *Caenorhabditis elegans* (see Antebi et al., 2000; Lee and Schroeder, 2012). Specifically, under favourable conditions (i.e., beneficial environmental temperature, stable food availability and low

population density), activated cGMP, TGF-β and insulin/IGF-1 signalling cascades trigger the biosynthesis of dafachronic acids via DAF-9/cytochrome P450, and the dafachronic acids activate the nuclear hormone receptor DAF-12 and promote continuous larval development in *Caenorhabditis elegans* (see Gerisch and Antebi, 2004; Motola et al., 2006; Schaedel et al., 2012). Under unfavourable conditions (i.e., high environmental temperature, limited availability of food and/or high population density), ascarosides are synthesised and inhibit cGMP, TGF-β and insulin/IGF-1 signalling cascades, suppressing dafachronic acids biosynthesis and DAF-12, leading to dauer formation (Hu, 2007; Fielenbach and Antebi, 2008). As the cGMP, TGF-β, insulin/IGF-1 and steroid hormone signalling pathways play crucial roles in dauer in *Caenorhabditis elegans*, they have been canonised as the "dauer pathways" or "dauer signalling pathways" (Stoltzfus et al., 2012; Gilabert et al., 2016).

1.2.3. The roles of microRNAs in the development of nematodes

In addition to signalling pathways and their components, microRNAs (miRNAs) also play crucial roles in the developmental processes of nematodes (Pasquinelli and Ruvkun, 2002; Vella and Slack, 2005; Ambros, 2011; Grishok, 2013); miRNAs are single-stranded RNA molecules which were first identified in *Caenorhabditis elegans* (see Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000). These short non-coding RNAs regulate gene expression at the post-transcriptional level (e.g., translation and degradation) (Bartel, 2004, 2009; Carthew and Sontheimer, 2009). In *Caenorhabditis elegans*, a range of miRNAs are recognised to govern developmental timing (Abbott et al., 2005; Boehm and Slack, 2005; Ren and Ambros, 2015). For instance, miRNAs of the *let-7*-family (e.g., *let-7*, and *mir-84* and *-241*), in particular, are closely associated with the nuclear hormone receptor DAF-12 in *Caenorhabditis elegans*, creating a feedback circuit to ensure robust developmental timing (see Bethke et al., 2009; Hammell et al., 2009; Hochbaum et al., 2011; Hunter et al., 2013). On the other hand, some miRNAs (*n* = 107) are differentially transcribed between stages of a continuous and an interrupted life cycle of *Caenorhabditis elegans*, 17 of which (within the *lin-4*, *let-7* and *mir-34* families) regulate development options (Karp et al., 2011). In contrast to the situation for *Caenorhabditis elegans*, roles of miRNAs in development have been explored only in a

select number of parasitic nematodes, including *Brugia malayi* and *Strongyloides ratti* (see Ahmed et al., 2013; Winter et al., 2015).

1.3. Resources and tools available to explore the developmental biology of parasitic nematodes

Explorations of the developmental biology of nematodes rely heavily on resources available in public databases, such as WormBook (http://www.wormbook.org), WormBase (https://www.wormbase.org/), WormBase ParaSite @ WormBase (https://parasite.wormbase.org/index.html), the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) and UniProt (https://www.uniprot.org). Such resources include published information as well as genomic, transcriptomic, proteomic and/or metabolomic data sets from studies published in the peer-reviewed literature. Such data sets are produced in the laboratory using advanced molecular technologies or tools (Metzker, 2010; Aebersold and Mann, 2016; Marshall and Powers, 2017) and assembled, annotated and analysed using computational methods (Kanehisa et al., 2017; The Gene Ontology Consortium, 2017; Fabregat et al., 2018; Korhonen et al., 2016; Korhonen and Gasser, 2018) to better understand biological processes and mechanisms. Just like the *genome* is the complement of DNA or genes of an organism or a developmental stage or tissue, the *transcriptome*, *proteome* and *metabolome* represent the RNA, protein and metabolite complements, respectively, with the suffix "*omic*" being an adjective and "*omics*" referring to the research or subject area for each of these complements (Ge et al., 2003; Yadav, 2007; Prohaska and Stadler, 2011).

Although molecular biological research of parasitic nematodes has not kept pace with the rapid advances in the *Caenorhabditis elegans* field, there has been some progress (Chuan et al., 2010; Gasser et al., 2016; Korhonen et al., 2016; Stoltzfus et al., 2017; International Helminth Genomes Consortium, 2019). To date, draft genomes are available for > 70 parasitic nematodes (WormBase ParaSite: https://parasite.wormbase.org/-index.html; Howe et al., 2017), such as *Brugia malayi*, *Ascaris suum*, *Trichinella spiralis*, *Loa loa*, *H. contortus*, *Trichuris suis*, *Necator americanus*, *Ancylostoma ceylanicum*, *Toxocara canis*, *Oesophagostomum dentatum* and *Strongyloides ratti* (e.g., Ghedin et al.,

2007; Jex et al., 2011; Mitreva et al., 2011; Desjardins et al., 2013; Schwarz et al., 2013; Foth et al., 2014; Jex et al., 2014; Tang et al., 2014; Schwarz et al., 2015; Zhu et al., 2015; Tyagi et al., 2015; Hunt et al., 2016). Particularly recent genome resequencing programs have significantly improved the quality of draft genomes of selected parasitic nematodes, such as *Ascaris suum* (see Wang et al., 2017) and *H. contortus* (see Doyle et al., 2018). Decoding the genomes provides some insights into the protein-coding genes, evolution and parasitism of selected parasitic nematodes (Dieterich and Sommer, 2009; Sommer and Streit, 2011; Hunt et al., 2016; Viney, 2017; International Helminth Genomes Consortium, 2019).

Achievements have also been made through the exploration of RNAs (mRNA and miRNA; transcriptomes) of parasitic nematodes, particularly for those of socioeconomic or veterinary importance, such as *Ancylostoma ceylanicum*, *Brugia malayi*, *Strongyloides stercoralis*, *H. contortus* and *Toxocara canis* (e.g., Datu et al., 2008; Choi et al., 2011; Stoltzfus et al., 2012; Laing et al., 2013; Schwarz et al., 2013, 2015; Ma et al., 2016; Zhou et al., 2017). Clearly, these explorations are improving our understanding of the molecular biology of parasitic nematodes and host-parasite interactions (Wang et al., 2011; Ansell et al., 2013; Heizer et al., 2013; Mangiola et al., 2013; Stoltzfus et al., 2017; Jex et al., 2019), and are enabling the discovery of novel intervention strategies (Manzano-Román and Siles-Lucas, 2012; Britton et al., 2015). However, there are still major knowledge gaps in the area of developmental biology of parasitic nematodes.

Some progress has been made in the proteomic analysis of parasitic nematodes, which has been focused primarily on molecules that might play crucial roles in the adaptation and parasitism of parasitic nematodes (Moreno et al., 2011; Soblik et al., 2011; Chehayeb et al., 2014; Wang et al., 2016; Liu et al., 2016; da Silva et al., 2018), but the proteomics of parasitic nematodes is still in its infancy. There are limited glycome and lipidome resources for parasitic nematodes (Joachim et al., 2000; Veríssimo et al., 2016; Jiménez-Castells et al., 2017), and there is a lack of metabolomic studies of these worms.

Relatively little progress has been made in the area of functional genomics of parasitic nematodes of animals. Genetic tools, such as RNA interference (RNAi) (Fire et al., 1998), have been attempted in nematodes including *Brugia malayi* (see Aboobaker and Blaxter, 2003; Song et al., 2010; Singh et al., 2012), *H. contortus* (see Geldhof et al., 2006; Kotze and Bagnall, 2006; Zawadzki et al., 2012) and *Ascaris suum* (see McCoy et al., 2015).

However, effective and reproducible knockdown has been achieved only in ascaridoids (McCoy et al., 2015), and there is no high throughput RNAi platform for parasitic nematodes comparable to those available for *Caenorhabditis elegans* (see Kamath et al., 2003; Lehner et al., 2006; Geldhof et al., 2007; Knox et al., 2007; Britton et al., 2012, 2016; Conte et al., 2015). The low efficacy and reproducibility of RNAi in most parasitic nematodes might relate to an absence of key components (SID-2; TSN-1; RDE-2; LIN-15B; RSD-3 and -6; ERI-3, -5, -6 and -7) from gene silencing pathways (Schwarz et al., 2013; Britton et al., 2016). Apart from RNAi, zinc-finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) technologies (Jinek et al., 2012; Hauschild-Quintern et al., 2013; Yi et al., 2014) might be considered for genome-scale functional analyses when/if conventional RNAi does not work effectively/efficiently (Ward, 2015, 2018; Britton et al., 2016; Sugi, 2016; Lok et al., 2017). Specifically, targeted mutagenesis has been achieved in *Caenorhabditis elegans*, *Pristionchus pacificus* and *Strongyloides stercoralis* using the CRISPR/Cas9 system (Cho et al., 2013; Witte et al., 2015; Gang et al., 2017). However, these genome editing tools have not yet been widely applied to any parasitic nematode of animals.

Therefore, although genomes, transcriptomes, proteomes and some glycomes have become available for parasitic nematodes, resources are not consistent with extensive data sets available for model organisms, such as *Caenorhabditis elegans*, which often prevents systems and developmental biological investigations of parasitic nematodes. The lack of detailed functional information for most parasitic nematodes also significantly constrains interpretations and conclusions about developmental processes in these worms.

1.4. Prospects for exploring developmental biology of parasitic nematodes

Compared with the free-living nematode *Caenorhabditis elegans*, there are major gaps in knowledge, molecular resources and functional genomic tools to explore the developmental biology of parasitic nematodes. Fortunately, comparative studies with well-characterised organisms, such as *Caenorhabditis elegans* and *Drospohila melanogaster*, can be used to understand the biology of parasitic worms (Bürglin et al., 1998; Gilleard, 2004; Hallem et al., 2007). *Caenorhabditis elegans* - arguably the best-

characterised and most tractable metazoan model organism (Corsi et al., 2015) - has a relatively close relationship with parasitic nematodes representing clades III and V (Blaxter et al., 1998; Bürglin et al., 1998; Blaxter and Koutsovoulos, 2015; International Helminth Genomes Consortium, 2019). Therefore, the information and knowledge available for *Caenorhabditis elegans* can be used for comparative studies in parasitic nematodes and to test hypotheses regarding conserved developmental processes.

A controversial hypothesis has been that dauer formation in *Caenorhabditis elegans* is a pre-adaptation to parasitism, and that the infective larva of parasitic nematodes represents a dauer-constitutive analogue (conveyed in the "dauer hypothesis"; cf. Hotez et al., 1993; Crook, 2014). Considering some physical and physiological similarities between free-living and parasitic nematodes, it is plausible that mechanisms controlling the developmental decisions of dauer-entry and -exit in free-living nematodes (dauer signalling pathway; section 1.2.2.) are similar to those controlling or responsible for the larval diapause and the activation of infective larvae in parasitic nematodes (Crook, 2014). This statement is supported by the presence of the development-associated hormone signalling module in both free-living (*Caenorhabditis elegans* and *Pristionchus pacificus*) and parasitic (Wang et al., 2009; Zhi et al., 2012) nematodes as well as the presence of respective *Caenorhabditis elegans* gene homologues (e.g., *daf-2*, *daf-7* and *daf-16*) in parasitic nematodes (e.g., Hu et al., 2010; McSorley et al., 2010; Li et al., 2014). Using a bioinformatic approach, Gilabert et al. (2016) explored the presence/absence of 47 signalling gene homologues across 24 nematode species, suggesting relative conservation in signalling in nematodes of clade III, IV and V, although no functional explorations were conducted. By using a comparative, transcriptomic approach, Stoltzfus et al. (2012) investigated the mRNA transcription of dauer signalling gene homologues in different developmental stages of *Strongyloides stercoralis* and *Caenorhabditis elegans*, and indicated a divergence in regulation of signalling between the two species, challenging the proposal of relative conservation in dauer signalling among nematodes (cf. Stoltzfus et al., 2014). Thus, the availability of genomic and transcriptomic resources for parasitic nematodes (section 1.3.) provides an opportunity to test aspects of the dauer hypothesis in comparative studies.

1.5. *Haemonchus contortus* - a suitable model to study developmental biology

Although being an excellent model for studying nematode biology, *Caenorhabditis elegans* is free-living nematode and cannot be used for exploring aspects of nematode biology pertaining to parasitism (Geary and Thompson, 2001). On the other hand, *Haemonchus contortus* (barber's pole worm; clade V) is an excellent model for parasitic nematodes of the order Strongylida – one of the largest groups of pathogenic worms of animals. *Haemonchus contortus* is an economically highly significant and very pathogenic blood-feeding (haematophagous) parasite of the abomasum of sheep, goats and other ruminants (see Gasser and von Samson-Himmelstjerna, 2016) with a direct life cycle and a high reproductive index, making it easy to maintain in experimental animals.

1.5.1. Developmental cycle

The life cycle of *H. contortus* (see Veglia, 1915) takes four weeks, and includes free-living stages in environment and parasitic stages in the host animal (Fig. 1.1): eggs are excreted in host faeces; the first-stage larvae (L1) develop inside the eggs, hatch and then moult to become second-stage (L2) and then third-stage (L3) larvae within approximately one week; after ingested via pasture by a ruminant host, the infective L3s moult (i.e., exsheath) and develop through fourth-stage larvae (L4s) to dioecious adults in the abomasum; female adults begin to produce eggs after mating with males. Specifically, the L3s of *H. contortus* can persist on pasture for weeks until being ingested and activated by host stimuli, including CO_2, pH and temperature, in the stomach of the host animal (Nikolaou and Gasser, 2006; Bekelaar et al., 2018a, 2018b). L4s can undergo arrested development (hypobiosis) in the host in response to seasonal, host and/or parasite factors (Blitz and Gibbs, 1971a, 1971b; Gibbs, 1982; Nikolaou and Gasser, 2006).

1.5.2. Haemonchosis and management strategies

The disease (haemonchosis) caused by *H. contortus* occurs in almost all tropical and subtropical regions of the world (O'Connor et al., 2006; Besier et al., 2016a). The distribution of this parasite and haemonchosis outbreaks depend largely on the

temperature and rainfall, as warm temperature and high humidity are essential for the free-living larvae to develop and survive. The ability of *H. contortus* to survive relies on the ability of L3s (infective larvae) and L4s to enter developmental arrest (hypobiosis within the host) to adapt to a range of climatic and environmental conditions (Waller and Chandrawathani, 2005; Besier et al., 2016a).

It is estimated that hundreds of millions of ruminants are affected by haemonchosis, causing substantial production losses globally (Gasser and von Samson-Himmelstjerna, 2016). An investigation carried out in New South Wales, Australia indicated that haemonchosis results in > 10% of annual mortalities in Merino ewes on farms in the absence of control programs (Kelly et al., 2010). Although highly significant, the negative economic impact of haemonchosis is challenging to assess/quantitate; *Haemonchus contortus* infection is often not monitored routinely on farms (Besier et al., 2016b). The control of haemonchosis (like other diseases caused by distinct strongylid nematodes) relies heavily on routine treatment with anthelmintics, such as moxidectin and monepantel, in spite of the availability of a commercial vaccine (Barbervax®) (Besier et al., 2016b; Nisbet et al., 2016).

The excessive and often uncontrolled use of anthelmintics has induced drug resistance in *H. contortus* and related strongylid nematodes around the world (Papadopoulos et al., 2012; Falzon et al., 2013; Tsotetsi et al., 2013; Playford et al., 2014; Chandra et al., 2015). Combined formulations of anthelmintics, such as mixtures of benzimidazoles, levamisole, macrocyclic lactones and/or closantel as well as the newly introduced derquantel+abamectin, are often used to increase the efficacy over a single product (Besier et al., 2016b). However, *H. contortus* can develop resistance to all current anthelmintics, including the relatively recently-introduced monepantel (Kaplan and Vidyashankar, 2012; Veríssimo et al., 2012; Mederos et al., 2014; van den Brom et al., 2015; Kotze and Prichard, 2016). Current evidence indicates that changes in genes, cell metabolism, target expression, substrate competition and drug efflux are associated with drug resistance development (Kotze and Prichard, 2016; Whittaker et al., 2016), but major knowledge gaps remain in this area (Kotze et al., 2014; Whittaker et al., 2016).

To achieve an effective control of haemonchosis and circumvent anthelmintic resistance issues, major efforts have been made to discover antigens that can induce effective immunoprotection. The immunogenic potential of excretory/secretory (ES)

products (Vervelde et al., 2003), whole-worm proteins (Yan et al., 2010), cuticular molecules (e.g., *Hc*-sL3) (Jacobs et al., 1999; Piedrafita et al., 2012, 2013) and intestinal extracts (e.g., H-gal-GP and H11) (Smith et al., 1994, 2003; Newton and Munn, 1999; Dicker et al., 2014) have been explored. High levels of immunoprotection have been achieved using the native, gut-derived membrane molecules H-gal-GP and H11 of *H. contortus*, a cocktail of such molecules now represents Barbervax® (the commercial vaccine), registered for use in lambs (Besier et al., 2012, 2015). However, this parasite-derived vaccine cannot induce long-lasting protection (Besier et al., 2015; Nisbet et al., 2016). DNA vaccination has also been assessed in goats against haemonchosis. For example, plasmid constructs encoding HC29 (Sun et al., 2011), H11 and caprine IL-2 (Zhao et al., 2012), glyceraldehyde-3-phosphate dehydrogenase (Han et al., 2012) or Dim-1 (Yan et al., 2013) were injected into goats, but did not achieve adequate protection (worm burdens being reduced by 35.6-51.1%) against *Haemonchus* to be promising vaccine candidates (see Nisbet et al., 2016).

1.5.3. Status of 'omics for H. contortus

A detailed understanding of the molecular biology (e.g., development and host-parasite interactions) of *H. contortus* could enable the discovery of new intervention targets, and assist in the development of anthelmintics and vaccines. This goal could be achieved using integrative 'omics where advanced nucleic acid sequencing, mass spectrometry and computational technologies are employed in combination (Cantacessi et al., 2012; Korhonen et al., 2016; Stoltzfus et al., 2017).

For *H. contortus*, some resources are available. In 2013, draft genomes were assembled and annotated for the McMaster and MHco3 ISE strains of *H. contortus* (see Laing et al., 2013; Schwarz et al., 2013), and provided new and useful insights into the genome structure and evolution (gene expansions/contractions), parasitism (e.g., inferred ES molecules), RNAi machinery (e.g., absence of critical components) and potential drug targets (e.g., kinase, phosphatase, GTPases, transporters, channels, transcription factors and GPCRs) in this parasitic nematode (see Schwarz et al., 2013; Gasser et al., 2016; Laing et al., 2016). Nonetheless, these draft genomes, assembled from short-read data sets obtained via Illumina sequencing, were fragmented. In order to address this issue of

fragmentation, Doyle et al. (2018) assembled an improved genome (~279 Mb) for *H. contortus* (MHco3 ISE strain) encoding 19,430 protein coding genes (BioProject no. PRJEB506). This significantly improved genome for *H. contortus* has provided major benefits for a range of genomic, informatic and molecular studies of this and related strongylid nematodes.

In parallel to the drafting of genomes, extensive transcriptome sequencing of *H. contortus* has been undertaken. Transcriptome resources are available for key developmental stages (eggs, larvae and adults) and both sexes (Haecon-5 and MHco3 ISE strains) and for the gut of female adults (MHco3 ISE strain) of *H. contortus* (see Laing et al., 2013; Schwarz et al., 2013; BioProject nos. PRJEB506 and PRJNA205202). These data sets have enabled studies of the changes and differences in transcription of protein-coding genes between or among the stages, sexes or tissues of this parasitic nematode (Laing et al., 2013; Schwarz et al., 2013; Gasser et al., 2016). Transcriptomic data sets are available also for artificially exsheathed L3s (xL3s) of *H. contortus* (Preston et al., 2017) and for small regulatory molecules, including 189 miRNAs and > 10,000 small interfering RNAs in L3 and adult stages, and 154 *piwi*-associated RNAs exclusively in the adult stage of *H. contortus* (see Winter et al., 2012).

Proteomic investigations of *H. contortus* have been undertaken also to explore ES proteins. In early studies (Yatsuda et al., 2003; Kuang et al., 2009), only small numbers of proteins were identified, primarily due to limited transcriptomic or genomic data sets (i.e., expressed sequence tags and contigs) being available at the time. However, the availability of draft genomes and transcriptomes (Laing et al., 2013; Schwarz et al., 2013) enables the high-throughput identification and quantitation of proteins in *H. contortus* using advanced mass spectrometry methods. For instance, recent investigations of intestinal protein extracts and ES products interacting with peripheral blood mononuclear cells (PBMCs) identified > 400 proteins, including aminopeptidases (H11), zinc metallopeptidases, glutamate dehydrogenase and apical gut membrane polyproteins, inferred to be immunogenic (Dicker et al., 2014; Gadahi et al., 2016), and one study (Wang et al., 2016) reported proteins being involved in the activation of the infective larvae of *H. contortus*. Despite these studies, no comprehensive somatic or ES proteomes have been defined for the distinct stages and sexes of *H. contortus*. Clearly, such

resources are needed if comprehensive molecular investigations of the developmental biology of *H. contortus* are to be undertaken.

1.5.4. Current status of knowledge of the developmental biology of H. contortus

Genomic, transcriptomic and proteomic approaches are enabling developmental biological investigations of *H. contortus*. Using 454 sequencing technology, Cantacessi et al. (2010) explored transcriptional differences between the free-living L3s and CO_2-activated L3s of *H. contortus*, and using mass spectrometry technology, Wang et al. (2016) investigated differential protein expression between the free-living L3s and NaOCl-activated L3s of this parasite. However, due to technical limitations, both of these studies achieved a low coverage of mRNAs and proteins of the developmental stages being studied. Using advanced next-generation sequencing (NGS) technology, Schwarz et al. (2013) compared transcriptomes between one stage and the next of *H. contortus* during development from egg to adult, revealing substantial changes in mRNA transcription during the transitions. This study inferred a range of molecules (e.g., kinases, phosphatases, GPCRs, transcription factors, peptidases and metabolism enzymes) to be involved in the development and/or parasitism of *H. contortus* (see Schwarz et al., 2013; Gasser et al., 2016). However, detailed information about the biological processes involved in development and parasitism of this parasitic nematode is still lacking due to a lack of functional information and molecular resources linked to a well-assembled and annotated genome.

Interestingly, some functional studies of individual genes of *H. contortus* have been explored (Wang et al., 2017). These genes include *Hc-daf-2*, *Hc-age-1*, *Hc-aap-1*, *Hc-pdk-1* and *Hc-daf-16*, which are homologues of those involved in the dauer-associated insulin-like signalling pathway of *Caenorhabditis elegans* (see Hu et al., 2010; Li et al., 2014a, 2014b, 2016). Homologues of such molecules have high transcription levels in the infective stage of *H. contortus* (see Hu et al., 2010; Li et al., 2014a, 2014b, 2016), suggesting key roles for the insulin-like signalling pathway in the infective L3s of this parasitic nematode.

Some findings provide evidence to support the "dauer hypothesis" (section 1.4.). Using genomic and transcriptomic resources available at the time, Mohandas et al. (2016)

reconstructed the insulin-like signalling pathway for *H. contortus* using a bioinformatic approach, providing a starting point for functional explorations of this pathway. Gilabert et al. (2016) explored the conservation of genes involved in cGMP, TGF-β, insulin-like and steroid hormone signalling (dauer) pathways in nematodes representing distinct clades, stimulating further work on insulin-like signalling and interconnected pathways that might play roles in the development of parasitic nematodes. Recently, He et al. (2018) investigated the roles of *Hc-tgfbr1* - a homologue of the TGF-β receptor-coding gene *daf-1* of *Caenorhabditis elegans* - in the developmental transition from the free-living to the parasitic stage of *H. contortus*. These published works all point toward the need to elucidate these pathways using integrative 'omic studies.

1.5.5. In vitro culture of H. contortus

The use of in vitro culture methods can strongly underpin 'omic studies. Many studies have focused on developing such methods for short-term culture outside of the host animal, with a focus on attempting to culture *Haemonchus* from egg to reproductively active adult. However, no study has managed to raise and maintain reproductively active adults in culture. Nonetheless, considerable progress has been made in maintaining parasitic larvae in culture for extended periods (1-2 weeks; cf. Preston et al., 2016)

In vitro methods have been developed for the activation (exsheathment) of larvae of *H. contortus*. Exsheathment is a process linked to the rupture and release of the outer sheath of L3s, which appears to be triggered by host stimuli and achieved by the secretion of proteases by the parasite (Rogers and Sommerville, 1957; Ozerol and Silverman, 1972; Petronijevic et al., 1986; Wharton, 1991). To understand the biological triggers involved in larval exsheathment, various in vitro techniques were assessed to achieve this developmental process (e.g., Lapage, 1933; Silverman and Podger, 1964; Slocome and Whitlock, 1970; Mapes, 1972). The third-stage larvae (L3s) are incubated in different "exsheathing media" at approximate host temperature (38 °C) with or without carbon dioxide (CO_2) (Coles et al., 1980; Conder and Johnson, 1996). The effects of sodium hypochlorite, sodium tetraborate, acid pepsin, bile, Earle's balanced salt solution (EBSS), nematode washing buffer (NBS) and/or CO_2 on larval exsheathment in vitro were compared; sodium hypochlorite was shown to achieve almost 100% exsheathment in

vitro, being the most efficient exsheathment approach (Coles et al., 1980; Conder and Johnson, 1996). In addition, CO_2 appears to be a requirement for larval exsheathment of *H. contortus* (see Bekelaar et al., 2018a), and a rapid increase in temperature (heat shock) has been recognised as the biological trigger for this process (Bekelaar et al., 2018b). Although in vitro techniques for exsheathment have enabled the explorations of molecular processes involved in the larval activation of *H. contortus* (see Cantacessi et al., 2010; Wang et al., 2016), little is known about development after exsheathment, because the in vitro-culture media used lacked nutrients required for development beyond xL3.

Preston et al. (2016) tackled this issue and established a system that could achieve larval exsheathment and development of *H. contortus* in vitro. In this system, L3s (stored at 10 °C for up to three months) were exsheathed using low concentration (0.15%) of sodium hypochlorite, then washed in physiological saline, and incubated in Luria Bertani (LB) medium at 38 °C with 10% CO_2 and 100% humidity. The motility and development (pharynx and width) of the exsheathed larvae in vitro can be assessed, facilitating high-throughput screening of compounds for inhibition of larval motility and development (cf. Preston et al., 2017; Jiao et al., 2017; Dilrukshi Herath et al., 2018). Because of the high exsheathment rate that this technique can achieve, it can be used to synchronise larval exsheathment and subsequent larval development in vitro, which is critical for integrative transcriptomic, proteomic and lipidomic investigations. Therefore, this established in vitro system provides a solid platform for detailed 'omic explorations of *H. contortus* during its developmental transition from the free-living to the parasitic larval stage.

1.6. Conclusions and research aims

In the past two decades, significant progress has been made in the sequencing, assembly, annotation and analyses of genomes and transcriptomes of parasitic nematodes of socioeconomic importance. This progress has somewhat improved our knowledge and understanding of these pathogens at the molecular level. However, compared with the free-living nematode *Caenorhabditis elegans*, the areas of functional genomics, transcriptomics, proteomics and lipidomics of parasitic nematodes are still in their infancy, and there are major gaps in our knowledge and understanding of the molecular

biology of parasitic nematodes. The information on signalling molecules, molecular pathways and miRNAs that are known to be involved in developmental processes in *Caenorhabditis elegans* and the availability of some molecular resources (draft genomes, transcriptomes and some proteomes) for selected parasitic nematodes provide a basis to start exploring the developmental biology of parasitic nematodes. Indeed, some studies have identified molecules and pathways that might associate with developmental processes in *H. contortus*. However, detailed information is scant and 'omics resources are still limited, preventing a proper integration of 'omic data sets and comprehensive analyses. Moreover, almost nothing is known about the functional roles of pheromones, hormones, signalling pathways and post-transcriptional/post-translational regulations in the development of key parasitic nematodes, including *H. contortus*, throughout their entire life cycles.

Although *Caenorhabditis elegans* is an excellent model to assist molecular studies of parasitic nematodes, its use is limited when it comes to explorations of infective larvae and the development of parasitic nematodes within host animals. A deep understanding of parasitic nematodes, such as *H. contortus*, requires substantially enhanced resources and the use of integrative 'omics approaches for analyses. The improved genome and well-established in vitro larval culture system for *H. contortus* provide unprecedented opportunities for comprehensive studies of the transcriptomes (mRNA and miRNA), proteomes (somatic, ES and phosphorylated proteins) and metabolomes (e.g., polar and neutral lipids) of this nematode. Such resources should enable in-depth explorations of its developmental biology at a level, not previously possible. The ***objective*** of this thesis was to substantially advance our knowledge and understanding of the developmental biology of *H. contortus* using genomic, transcriptomic, proteomic, lipidomic and informatic studies, in order to assess an integrative multi-omics approach for the exploration of parasitic nematodes more generally.

The *specific research aims* of this project were:

(1) To establish comprehensive transcriptomic, proteomic and lipidomic resources for different developmental stages/sexes of *H. contortus* for profound molecular investigations (Chapters 2-4);

(2) To explore alterations in transcription and/or protein expression during the developmental transition from free-living to parasitic larvae of *H. contortus* in vitro (Chapter 5);

(3) To define the dauer-like signalling pathways in *H. contortus* based on knowledge and resources available for *Caenorhabditis elegans* (Chapter 6);

(4) To elucidate the dauer-like signalling pathways and the involvement of bile acid-like dafachronic acids in the development of *H. contortus* (Strongylida) and/or other nematodes, particularly in the developmental transition from the free-living to the parasitic phase (Chapters 7 and 9).

1.7. References

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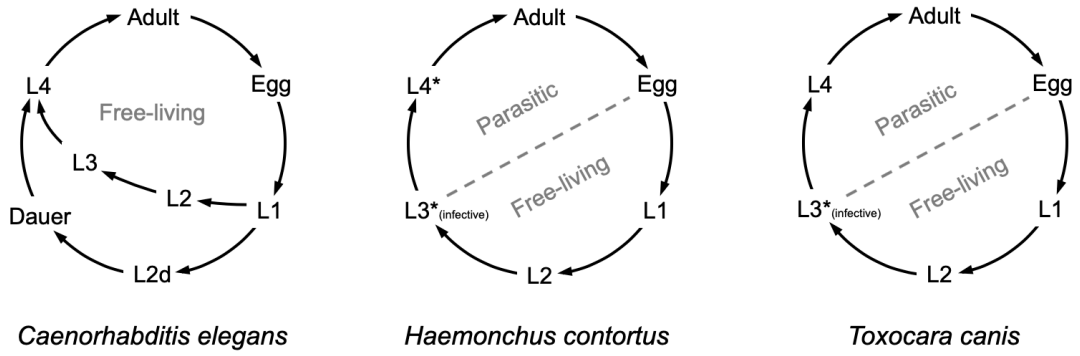


Fig. 1.1. Schematic diagram of life cycles of *Caenorhabditis elegans* (free-living nematode), *Haemonchus contortus* and *Toxocara canis* (parasitic nematodes). *Caenorhabditis elegans* has a direct life cycle: individual first-stage larvae (L1s) develop inside eggs, hatch and then develop to second (L2), third (L3), fourth (L4) -stage larvae and reproductively active adults. Larvae at the late L1 stage can undergo diapause at the second moult (L2d) to become "dauer" larvae in response to unfavourable conditions; development resumes when favourable conditions return (Hu, 2007). *Haemonchus contortus* has both free-living and parasitic phases in its life cycle: Individual L1s develop inside eggs, hatch and develop to L2s and (infective) L3s stages in the environment; the infective L3s developmentally arrest on pasture until ingested by a host, after which they then develop to L4s and adult stages in the stomach (abomasum) of the host. L4s can undergo arrested development (hybiosis) in response to seasonal and/or host immune or physiological conditions (Nikolaou and Gasser, 2006). *Toxocara canis* develops via the egg to L1, L2 and then L3 (within egg) in the environment. Following ingestion by a dog (puppy of < 12 weeks of age), infective L3s emerge from eggs in the duodenum, undertake hepato-pulmonary migration and then return to the small intestine, where they develop to L4s and adults. In dogs of > 12 weeks of age, following hepato-pulmonary migration, L3s migrate to tissues and undergo developmentally arrest. In the male dog, this is a dead-end for the parasite; in female dogs, these larvae can activate in the last trimester of pregnancy and undergo transplacental transmission (99%) and/or then transcolostoral transmission (1%) postpartum (Schnieder et al., 2011). Asterisk indicates developmental variant in *H. contortus* and *Toxocara canis*.

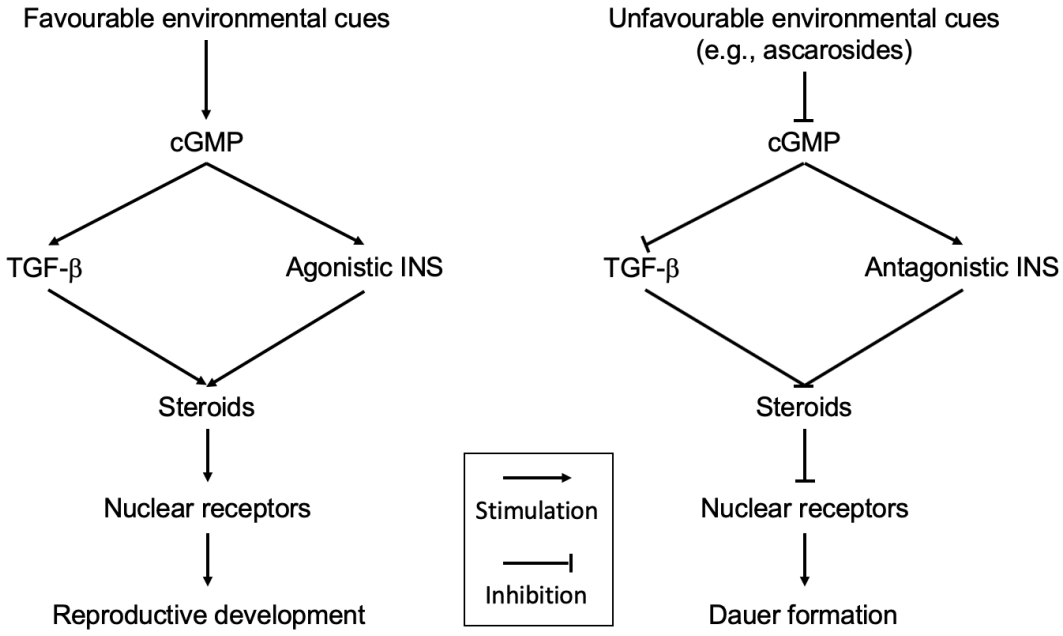


Fig. 1.2. Schematic diagram of the dauer signalling cascade governing reproductive development and dauer formation in *Caenorhabditis elegans*. *Abbreviations:* cGMP: cyclic guanosine monophosphate; TGF-β: transforming growth factor-β; INS: insulin-like peptide.

Chapter 2. Somatic proteome of *Haemonchus contortus*

Abstract

Currently, there is a dearth of proteomic data to underpin fundamental investigations of parasites and parasitism at the molecular level. Here, using a high throughput LC-MS/MS-based approach, we undertook the first comprehensive, large-scale proteomic investigation of the barber's pole worm (*Haemonchus contortus*) - one of the most important parasitic nematodes of livestock animals worldwide. In total, 2487 unique *H. contortus* proteins representing different developmental stages/sexes (i.e., eggs, L3s and L4s, female (Af) and male (Am) adults) were identified and quantified with high confidence. Bioinformatic analyses of this proteome revealed substantial alterations in protein profiles during the life cycle, particularly in the transition from the free-living to the parasitic phase, and key groups of proteins involved specifically in feeding, digestion, metabolism, development, parasite-host interactions (including immunomodulation), structural remodelling of the body wall and adaptive processes during parasitism. This proteomic data set will facilitate future molecular, biochemical and physiological investigations of *H. contortus* and related nematodes, and the discovery of novel intervention targets against haemonchosis.

2.1. Introduction

Parasitic worms cause substantial mortality and morbidity in animals, and major losses to food production worldwide. Roundworms (= nematodes) cause destructive diseases that affect hundreds of millions of livestock animals (e.g., sheep, goats and cattle), resulting in economic losses of tens of billions of dollars per annum globally (Roeber et al., 2013; Gasser and von Samson-Himmelstjerna, 2016). Despite substantial efforts to control these worms, commercial vaccines are lacking, and treatment relies heavily on only a relatively small number of drugs (anthelmintics). As drug resistance is now widespread, there is an urgent need to develop new and effective interventions, built on a solid understanding of these worms, their relationship with animal host(s) and parasitism at the molecular level.

Advanced nucleic acid sequencing and bioinformatic technologies have enabled an unprecedented number of worm genomes to be decoded (e.g., Schwarz et al., 2013; Laing et al., 2013; Tang et al., 2014; Schwarz et al., 2015; Tyagi et al., 2015; Korhonen et al., 2016). Although draft genomes provide investigators with resources to explore these worms at the molecular level, the expression profiles and functions of most parasite proteins are unknown. Researchers have begun using genomic sequence data sets to assist investigations of the expression, localisation and function of genes employing advanced transcriptomic and proteomic tools.

While transcriptomics can quantify RNAs, such as messenger and small RNAs, advanced proteomics provides a means of identifying and quantifying proteins in whole worms, dissected tissues or particular developmental stages (Bennuru et al., 2011; Morris et al., 2015). The digital resources (‘tool-kits’) to investigate and mine genomic and transcriptomic data sets are now available. The databases NEMBASE (Parkinson et al., 2004) and Nematode.net (Martin et al., 2015) contain relatively extensive genomic and/or transcriptomic datasets, and ParaSite (Howe et al., 2016) provides a wealth of information and features in WormBase for key parasitic worms. However, a critical appraisal of current literature reveals that, with the exception of selected filarioid nematodes (Bennuru et al., 2011; Armstrong et al., 2016), comprehensive proteomic data sets are scant for most other socio-economically important parasitic nematodes, despite the massive technological advances made in recent years in the field of proteomics (Zhang et al., 2013; Aebersold and Mann, 2016).

The aim of the present study was to define the proteome of one of the most important parasitic nematodes of livestock animals worldwide, *Haemonchus contortus*, using high throughput liquid chromatography–mass spectrometry (LC-MS/MS), and explore biological pathways involved in parasitism employing advanced informatics and current genomic/transcriptomic resources (cf. Schwarz et al., 2013; Preston et al., 2017).

Haemonchus belongs to one of the largest orders of nematodes (Strongylida) which adversely impact on the health and wellbeing of animals (Gasser and von Samson-Himmelstjerna, 2016). It is a blood-feeding (haematophagous) worm of the stomach (abomasum) of ruminants, causing a disease, called haemonchosis, leading to anaemia and/or associated complications, ill thrift and death in severely affected animals (Gasser and von Samson-Himmelstjerna, 2016). This worm is transmitted orally from contaminated pasture to the host through a direct life cycle: eggs are excreted in host faeces; the first-stage larva (L1) develops inside the egg to then hatch (within 1 day) and develop through to the second- and third-stage larvae (L2s and L3s) in about a week; the infective L3s are then ingested by the host, exsheath (xL3) and develop via fourth-stage larvae (L4) to dioecious haematophagous adults (within 3 weeks) in the stomach (Veglia, 1915). Given the scant proteomic data available for parasitic nematodes (i.e., cuticlar proteins, excretory/secretory proteins and extracellular vesicles) (Yatsuda et al., 2003; Hewitson et al., 2008; Wang et al., 2013; Buck et al., 2014; Chehayeb et al., 2014; Liu et al., 2015; Zamanian et al., 2015; Tzelos et al., 2016; Sperotto et al., 2017; Stoltzfus et al., 2017), defining the first proteome representing key developmental stages of *H. contortus* will facilitate elucidating aspects of its unique biology and its ability to survive and maintain a complex relationship with its host animal, which could open the door to discovering new interventions against this and related worms.

2.2. Materials and methods

2.2.1. Parasite stages

Haemonchus contortus was produced in Merino lambs (6 months of age; Victoria, Australia), maintained under helminth-free conditions (animal ethics approval no. 1714374, The University of Melbourne). Different developmental stages/sexes of *H.*

contortus - i.e., eggs, L3s and L4s, female (Af) and male (Am) adults - were produced as described previously (Campbell et al., 2008; Schwarz et al., 2013). Briefly, sheep were inoculated intra-ruminally (via oral intubation) with 7000 infective L3s of *H. contortus*. Eggs were isolated from the faeces from infected sheep (1 month after inoculation) using a sucrose flotation procedure (Mes et al., 2007). L3s were produced in culture as described previously (Nikolaou et al., 2002). L4s and adults (Af and Am) were collected from the abomasa of infected sheep 9 and 28 days after the inoculation of sheep with L3s, respectively. Four biological replicates of each developmental stage were prepared, washed extensively (five times) in 50 ml volumes of physiological saline (pH 7.0), pelleted and then frozen at - 80 °C until further analysis.

2.2.2. Protein extraction

Proteins were extracted from each of four replicates of each egg, L3, L4, Af and Am. In brief, ~30,000 eggs or larvae, or 100 adults were transferred to sterile (1.5 mL) Eppendorf tubes containing 500 µL of lysis buffer (8 M urea in 100 mM triethyl ammonium bicarbonate, pH 8.5). Each sample was then subjected to three freeze (-196 °C) - thaw (37 °C) cycles (Caito and Aschner, 2015) and centrifuged at 10000 ×*g* for 30 sec, ultra-sonicated (20 kHz) using a BioRuptor (10 cycles: 30 sec on - 30 sec off) on ice. Each sample was supplemented with protease inhibitor cocktail set I (Merck, Denmark) and incubated at 23 °C for 30 min. Then, samples were centrifuged at 12000 ×*g* for 30 min, and the supernatants collected for analyses. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

2.2.3. In-gel and in-solution digestion, and LC-MS/MS analysis

The analysis of the proteome of *H. contortus* was conducted using in-gel and in-solution digestion protocols, as described previously (Ang et al., 2011). For the in-gel procedure, samples containing proteins from the egg, L3, L4, Af *and* Am stage (30 µg of each) were resolved in a pre-cast NuPAGE 4-12% Bis-Tris gel (Invitrogen, USA) at a 150 V (constant) for 1 h. The gel was stained with Coomassie blue for 10 h (shaking) and destained with H_2O. The gel lane was sliced into 10 equal pieces, reduced with 10 mM

tris (2-carboxyethyl) phosphine (TCEP) at 55 °C for 45 min, then alkylated with 55 mM iodoacetamide in the dark at 22 °C for 30 min, followed by a digestion with sequencing-grade, modified trypsin (Pierce, USA) at 37 °C for 16 h. Samples were spiked with Hyper Reaction Monitoring (HRM) calibration peptides (Biognosys, Switzerland) prior to LC-MS/MS analysis.

For the in-solution procedure, samples containing proteins (100 µg) from either the egg, L3, L4, Af *or* Am stage were reduced with 10 mM tris (2-carboxyethyl) phosphine (TCEP) at 55 °C for 45 min, then alkylated with 55 mM iodoacetamide in the dark at 22 °C for 30 min, followed by a double-digestion with Lys-C/trypsin mix (Promega, USA) at 37 °C for 16 h (4 h for Lys-C digestion and 12 h for trypsin digestion). The tryptic samples were acidified with 1.0% (v/v) formic acid, purified using Oasis HLB cartridges (Waters, USA). Proteins in these samples were divided into eight fractions using the high pH reversed-phase peptide fractionation kit (Pierce, USA) according to the manufacturer's protocol. All fractions were freeze-dried prior to re-suspension in aqueous 2% w/v acetonitrile and 0.05% trifluoroacetic acid (TFA) w/v, and were spiked with HRM calibration peptides (Biognosys, Switzerland) before LC-MS/MS analysis.

2.2.4. Data-dependent LC-MS/MS

For data-dependent acquisition (DDA) profiling, tryptic peptides were analysed using the Q Extractive Plus Orbitrap and Fusion Lumos Orbitrap mass spectrometers (Thermo Fisher, USA), in order to achieve highest possible proteome coverage in relation to the *H. contortus* genome. The LC system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 µm x 50 cm). The tryptic peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 2% v/v CH_3CN containing 0.1% v/v formic acid for 6 min, applied before the enrichment column was switched in-line with the analytical column. The eluents were 0.1% v/v formic acid (solvent A) and 100% v/v CH_3CN in 0.1% v/v formic acid (solvent B). The gradient was at 300 nl/min from (i) 0-6 min at 3% B; (ii) 6-95 min, 3-20% B; (iii) 95-105 min, 20-40% B; (iv) 105-110 min, 40-80% B; (v) 110-115 min, 80-80% B; (vi) 115-117 min 85-3% and equilibrated at 3% B for 10 min before injecting the next sample. The Q Extractive Plus mass spectrometer

was operated in the data-dependent mode, whereby full MS1 spectra were acquired in a positive mode, 70000 resolution, AGC target of $3e^6$ and maximum IT time of 50 ms. Fifteen of the most intense peptide ions with charge states of ≥ 2 and intensity thresholds of ≥ $1.7e^4$ were isolated for MSMS. The isolation window was set at 1.2 m/z, and precursors fragmented using a normalised collision energy of 30, a resolution of 17500, an AGC target of $1e^5$ and a maximum IT time of 100 ms. Dynamic exclusion was set at 30 sec. The Fusion Lumos mass spectrometer was operated in positive-ionisation mode, with the spray voltage set at 1.9 kV and the source temperature at 275 °C. The mass spectrometer was operated in the data-dependent acquisition mode, whereby full MS1 spectra were acquired in a positive mode at 120000 resolution, with an AGC target of $5e^5$. The "top speed" acquisition method mode (cycle time: 3 sec) on the most intense precursor was used, whereby peptide ions with charge states of 2 to 5 were isolated using an isolation window of 1.2 m/z and fragmented with a high energy collision (HCD) mode employing a stepped collision energy of 30 ± 5%. Fragment ion spectra were acquired in Orbitrap at 15000 resolution. Dynamic exclusion was activated for 30 sec.

2.2.5. Data-independent LC-MS/MS

Data independent acquisition (DIA) was carried out on the Q Extractive Plus mass spectrometer using the HRM-DIA methodology (Bruderer et al., 2015). Here, a survey scan from 400-1000m/z at 70000 resolution and an AGC target of $3e^6$ at 50 ms was followed by 30, 21 m/z DIA windows at 17500 resolution, AGC of $1e^6$ and the IT time set at "auto". Stepped normalised collision energies were set at 28, 30 and 32. All spectra were acquired in the profile mode.

2.2.6. Protein identification and quantification

Protein/peptide identification and subsequent spectral library generation were conducted using Proteome Discoverer (v.2.1, Thermo Fischer Scientific) with the Sequest HT search engine and the Percolator semi-supervised learning algorithm (Kall et al., 2007). An in-house sequence database, with GenBank's non-redundant (NR) protein database annotation, was established for *H. contortus* (16093 protein entries) based on

transcriptomes (Schwarz et al., 2013; Preston et al., 2017), PacBio long sequence reads (Bioproject: PRJEB2252 at NCBI) and additional, curated protein data for *H. contortus* (494 protein entries) (Mohandas et al., 2015; Stroehlein et al., 2015; Mohandas et al., 2016) as well as the Biognosys iRT peptide sequence. Search parameters were: a precursor tolerance of 20 ppm, MSMS tolerance of 0.05 Da, fixed modifications of carbamidomethylation of cysteine (+57 Da) and methionine oxidation (+16 Da). Peptides were accepted based on a false discovery rate (FDR) of < 0.01 at both the peptide and protein levels. For stage-specific identification and relative quantification comparisons, only protein identifications with ≥ 2 peptides and present in ≥ 3 biological replicates of at least one developmental stage were accepted.

HRM-DIA-based quantitation was carried out using Spectronaut software (Biognosys, v.11). The spectral library used for database search contained 3746 protein groups (34282 peptides), generated from a total of 65 in-gel-digested and basic reverse phase-separated peptides DDA experiments from the Q Extractive Plus mass spectrometer workflow. To achieve optimal quantitation, we used instrument-specific libraries, and employed the same chromatographic conditions. Results were exported and analysed in Spectronaut using default settings and a stringent *q*-value cut-off (< 0.01). For each protein, three peptides with the highest intensities were used for quantitative analysis. The data were normalised based on the median protein intensity under each condition. Fold-change thresholds of ≥ 2, with the inferior *q*-value set at ≤ 0.05, were used to establish whether a protein was up- or down-regulated. The *p*-values were adjusted using a Benjamini-Hochberg correction. Results are available via the PRIDE data repository (accession number: PXD009956).

2.2.7. Bioinformatic analyses of data sets

The UniProt repository was used for protein annotation (cellular compartment, subcellular location, transmembrane region or molecular function). Molecular functions of proteins predicted from the genome of *H. contortus*, or identified or quantified in the proteome of *H. contortus* were classified according to Gene Ontology (GO) using the program InterProScan (Mitchell et al., 2015). Multi-scatter plots were drawn using Perseus software (v.1.6.1.1) using default settings (Tyanova et al., 2016). Principal

component analysis (PCA) and hierarchical cluster analysis (HCA) were conducted in the R language using software packages Glimma v.1.2.1 and gplots v.3.0.1. The statistical significance of correlation analyses was set at $P < 0.05$. Biological functions were assigned to differentially expressed proteins using the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al., 2016). KEGG pathway annotation was conducted employing KEGG BLAST hits (E-value: $< 10^{-5}$) and corresponding KEGG Orthology (KO) terms (Mao et al., 2005). KO terms were then assigned to KEGG pathways and KEGG BRITE orthologous protein families by mapping these terms to the KEGG Orthology Based Annotation System (KOBAS) database (Xie et al., 2011). Enriched KEGG pathways were identified using a cut-off of $P < 0.01$ (Fisher's Exact test). KEGG functional enrichments of differentially expressed proteins were integrated and displayed using the program FuncTree (Uchiyama et al., 2015). Particular sequences, such as those of collagens and cuticular proteins, were compared with those available in WormBase (Howe et al., 2016) using blastp. Protein expression linked to lipid metabolism, expressed as total ion abundance of lipids ($n = 554$), was calculated in different stages/sexes of *H. contortus* from a recently published, label-free relative quantitation lipidome analysis data set (see detailed methods Chapter 4). A one-way ANOVA test was performed for multiple group comparisons using GraphPad Prism 6.0 software (GraphPad, La Jolla, USA). Statistical significance was set at $P < 0.05$.

2.3. Results

2.3.1. The somatic proteome of H. contortus

The proteome of *H. contortus* (Haecon 5 strain) was inferred from in-gel digested and basic reverse phase-separated peptides from key developmental (i.e., egg, L3, L4 and adult) stages of *H. contortus*. Amongst 62,080 peptides (with lengths of between 6 and 47 amino acids), we identified 4071 protein groups (Supplementary Table S2.1). Of 1946 (47.8%) orphan proteins (with unknown identity and function) identified, 99.8% ($n = 1942$) had relatively conserved homologs in various secernentean nematodes (e.g., *Ancylostoma ceylanicum*, *Angiostrongylus cantonensis*, *Ascaris suum*, *Brugia malayi*, *Caenorhabditis* spp. and *Loa loa*). This represents 24.5% of the proteins predicted from

the in-house sequence database of *H. contortus* (see Section 2; cf. Schwarz et al., 2013; Mohandas et al., 2015; Stroehlein et al., 2015; Mohandas et al., 2016; Preston et al., 2017).

2.3.2. Quantification of proteins in different developmental stages of H. contortus

From the somatic proteome, we used peptides identified only from the Q Extractive Plus mass spectrometer in order to construct an instrument specific spectral library for quantitative analysis employing the HRM-DIA method (Bruderer et al., 2016). This library consists of 3746 unique proteins and 34,282 peptides. A total of 2487 unique proteins representing all key developmental stages/sexes (i.e., egg, L3, L4, Af and Am) of *H. contortus* were identified and quantified with high confidence in the spectral library based on stringent criteria. The greatest number of proteins identified was in the egg stage (n = 1744), followed by L4 (n = 1697), Af (n = 1677) and Am (n = 1604), followed by L3 (n = 1208). The full list of proteins identified in each developmental stage is given in Supplementary Table S2.2. The comparisons of the identified proteins amongst different developmental stages of *H. contortus* are shown in a Venn diagram (Fig. 2.1A). Overall, most proteins (n = 1915, 77.0%) were shared by at least two developmental stages of *H. contortus*. Of these shared proteins, 753 (30.3%) were detected in all developmental stages studied.

To gain insights into parasitic stages (i.e., L4, Af and Am), 415 stage-specific proteins were identified. Conspicuous among them were molecules such as collagens and cuticular proteins (n = 10), nematode SCP/TAPS proteins (n = 19) and cysteine peptidases (n = 15). Within a particular developmental stage, the relative ratios of proteins expressed in individual stages ranged from 1.4% to 21.6%. Notably, the largest stage-specific set of proteins identified was in the egg (n = 376, 21.6%), followed by the free-living L3 stage (n = 106, 8.8%), whereas smaller numbers of proteins were enriched in the parasitic stages [L4 (n = 43, 2.5%), Af (n = 25, 1.5%) and Am (n = 22, 1.4%)].

The distribution of the molecular functions (GO level 2) of proteins identified in each stage, according to the GO, is summarised in Fig. 2.1B. Most proteins were associated with binding (GO: 0005488; 45.1-50.5%) and catalytic activity (GO: 0003824; 36.4-40.5%) in individual stages. Each of these two major functional categories (binding and

catalytic activity) contained at least 490 annotated proteins, whereas proteins involved in structure, transporter and antioxidant activities were less represented. A closer appraisal of molecular functions (GO level 3) revealed proteins involved predominantly in the binding of compounds, ions, other proteins and small molecules, while hydrolase molecules were represented mainly by the 'catalytic activity' category (Supplementary Table S2.3). In terms of the percentages of proteins classified in each sub-category, there was no obvious difference among the developmental stages studied (Fig. 2.1B).

2.3.3. Comparison of molecular functions

A comparison of the molecular functions inferred for (i) proteins predicted from the genome, (ii) proteins identified in the proteome and (iii) proteins quantified in the proteome revealed a relative concordance in the proportions (percentages) of proteins present in particular functional groups (Fig. 2.1C; Supplementary Table S2.4). For all three datasets/analyses (i-iii), there was concordance in the percentages of predicted or identified proteins associated predominantly with activities of molecular carriers (0.01-0.04%), cargo receptors (0.04-0.05%), antioxidants (0.3-0.6%), transcription regulators (0.8-1.8%), molecular function (1.5-1.9%), structural molecules (3.2-5.3%) and transporter (4.2-5.8%), with binding (48.4-50.3%) and catalytic (35.4-38.3%) activities dominating. The only discrepancy seen related to 'molecular transducer activity' which was underrepresented in proteins identified (0.7%) and quantified (0.6%) in the proteome compared with those predicted from the genome (2.9%), indicating limited or no expression of some genes in the *H. contortus* genome, or expression levels that were not detectable using the present proteomic approaches.

2.3.4. Alterations in protein expression during development

Principal component analysis (PCA) of the proteome of the distinct developmental stages/sexes of *H. contortus* was performed (Fig. 2.2A). The two-dimensional PCA showed that parasitic stages (i.e., L4, Af and Am) clustered tightly together, to the exclusion of the free-living stages (i.e., egg and L3). Interestingly, the difference between the two free-living stages was substantial. Following PCA analysis, a hierarchical

clustering showed a clear division of the proteomic data set into five distinct groups, corresponding to the different developmental stages of *H. contortus* (Fig. 2.2B). Moreover, together with multi-scatter plots (Fig. 2.2C), hierarchical clustering showed that the difference in expression within a particular stage (i.e., among the four replicates) was markedly less than differences among stages, allowing further comparisons of relative protein expression alterations during *H. contortus* development.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that proteins expressed differentially between or among at least two developmental stages were involved in one to five biological categories (i.e., cellular processes, environmental information processing, genetic information processing, organismal systems and metabolism), covering key aspects of parasite growth and metabolism (Supplementary Table S2.5). In the egg stage, differentially expressed proteins were mainly linked to genetic information processing (n = 478 of 504), whereas only a limited number of differentially expressed proteins associated with metabolism (n = 14) and/or cellular processes (n = 12) (Fig. 2.3A). In the genetic information processing category, translation (n = 182; ribosome, RNA transport, aminoacyl-tRNA biosynthesis and mRNA surveillance pathway) and transcription (n = 126; spliceosome) were the two major processes in the egg stage, whereas 77 proteins were inferred to relate to replication and repair processes (i.e., DNA replication, mismatch repair, nucleotide excision repair and base excision repair). Two additional, enriched pathways [environmental information processing (n = 52) and organismal systems (n = 130)] were represented in the L3 stage, to the exclusion of eggs (Fig. 2.3B; Supplementary Table S2.5). Unlike the egg stage, only a limited number (n = 26) of differentially expressed proteins was involved in the genetic information processing pathway in the L3 stage. Notably, more than 350 of 715 proteins expressed in L3s were enriched in metabolic pathways, including carbohydrate metabolism (n = 111; citrate cycle, glycolysis/gluconeogenesis, propanoate metabolism, glyoxylate and dicarboxylate metabolism, pyruvate metabolism and butanoate metabolism) as well as amino acid (n = 70), energy (n = 50) and lipid (n = 23) metabolism. Similar to the L3 stage, most differentially expressed proteins (n = 101 of 203) in the L4 stage were enriched within the metabolism category (Fig. 2.3C). This category was represented predominantly by amino acid (n = 58) and carbohydrate (n = 21) metabolism. The remaining categories of proteins specific to the L4 stage were inferred to be

associated with genetic information processing (n = 79), organismal systems (n = 20) and environmental information processing (n = 3). For the adult stages, in addition to metabolism (n = 299), which was also a major biological category, genetic information processing (n = 34) and organismal systems (n = 14) were two categories represented by proteins enriched in Af (Fig. 2.3D), but this was not the case for Am. Only four differentially expressed proteins were detected in Am, all of which were inferred to be involved in carbohydrate metabolism (Supplementary Table S2.5).

2.3.5. Lipid ion abundance

In total, 554 unique lipid species represented 4 lipid categories (i.e., glycerolipids, glycerophospholipids, sphingolipids and sterol lipids) extracted from equal amounts (3 mg per stage) of freeze-dried nematode material were used to calculate the total ion abundance of lipids in different developmental stages/sexes of *H. contortus* (see Fig. 2.4; Supplementary Table S2.6). Abundance was highest in the free-living L3 stage, followed by Af (which contain eggs) and eggs (similar abundance), followed by L4 and Am (with lowest abundance compared with L3; $P < 0.01$). Notably, total ion abundance was markedly higher in Af than in Am and the L4 stage.

2.4. Discussion

By defining a comprehensive proteome from body tissues of key developmental stages (egg, L3, L4 and adult) and both sexes (adult) of *H. contortus*, we have been able to provide the first insights, at the protein level, into key biological processes and pathways enriched in stages of this pathogen that likely underpin parasitism in the host animal. In the developmental cycle of *H. contortus* and related strongylid nematodes, the infective (L3) stage is ingested, and the parasitic stages establish in the stomach (abomasum) of the ruminant, feed on blood and other tissues and, rapidly grow, develop and reproduce within a period of three weeks. During this transition, one expects strictly controlled patterns of expression of particular protein groups relating to feeding, the acquisition of essential nutrients, metabolism as well as body structure and integrity, to meet major demands for worm growth and development within the host animal, and molecular

processes (including host cross-talk and immune modulation) that ensure the survival of the parasite in a harsh, gastric environment (parasitism). These patterns are clearly reflected in the pathways enriched in the somatic proteome of *H. contortus*.

Although the results of the proteomic analysis of the free-living stages of *H. contortus* was relatively inconspicuous (Supplementary Table S2.2), this was not the case for the parasitic stages. The findings suggest that the L4s and adults (parasitic) rely heavily on the catabolism of haemoglobin for survival once established in host, like other haematophagous parasites (Tort et al., 1999; Williamson et al., 2003). Peptidases, including aspartic peptidases, cysteine peptidases and metallopeptidases, are recognised as crucial molecules in the degradation of blood components, tissues and in anticoagulation, and are likely to be essential for growth, development and survival of *H. contortus* (see Williamson et al., 2003). Peptidases C1A (cysteine peptidases; $n = 15$), A1 (aspartic peptidases; $n = 9$), M12 (metallopeptidases; $n = 3$) and M13 (metallopeptidases; $n = 2$) were particularly abundant in the parasitic stages (Supplementary Table S2.2), in accord with transcriptomic data (Schwarz et al., 2013).

Pathway enrichment analysis highlighted the distinct metabolism (i.e., the proportion of enriched proteins dramatically increased from 3% in egg to more than 50% from the L3 stage onwards), reflecting the increasing demand for essential energy for their rapid growth during the transition from the free-living to parasitic phase of the parasite's life cycle. Enriched pathways included carbohydrate, amino acid, energy and lipid/fatty acid metabolism (cf. Fig. 2.3). These findings are in accord with previous results for the developmental transcriptome of *H. contortus* (see Schwarz et al., 2013), and suggest that metabolism is under tight post-transcriptional control in this haematophagous nematode. Unlike the relatively well controlled lipid metabolism in the developmental stages of *Caenorhabditis elegans* (Cooper and van Gundy, 1971; Ashrafi, 2007), lipid metabolism is down-regulated in *H. contortus* once this nematode reaches the parasitic stage. Here, we identified 23 relatively abundantly expressed proteins involved in the fatty acid degradation pathway in the L3 stage, whereas no enrichment in lipid-related metabolism was detected in parasitic stages (i.e., L4, Af and Am). This finding is consistent with the alterations observed in total lipid ion abundances in different stages of *H. contortus* (see Fig. 2.4; Supplementary Table S2.6), in which we recently indicated that *H. contortus* alters its lipidome to adapt to an 'opportunistic' way of life in the host animal (Wang et

al., 2018). We hypothesise that *Haemonchus* tends to down-regulate its lipid metabolism (mainly energy storage lipids - triradylglycerols) to rely on a supply of corresponding fatty acids from blood and tissues once established at its predilection site (abomasum) in the host animal (Harder, 2013; Wang et al., 2018). Whether similar processes are active in other haematophagous nematodes is presently unknown and warrants future investigation.

Haemonchus contortus develops from infective L3s to reproductively active adults within three weeks. This parasitic phase is associated with rapid growth and development; a panel of at least 8, 14, 8 and 12 collagens and cuticular proteins are represented in the proteome of the L3, L4, Af and Am stages, respectively, whereas five are in the egg stage. Although there are differences in the numbers of predicted collagens between free-living stages (n = 7) and parasitic stages (n = 4-7), seven of them are common to L4, Af and Am (Supplementary Table S2.7). Such proteins are expected to be critical for the maintenance of body form, integrity, and for contact with the host interface and environment. An analysis of relative abundance indicated that these collagens and cuticular proteins are expressed mainly in the L4 and adult stages (Supplementary Table S2.7), coinciding with a peak of expression of prolyl 4-hydroxylase (Supplementary Table S2.2), a key enzyme involved in collagen synthesis and assembly. All 22 collagens and cuticular proteins identified have significant sequence similarity (23-73%) to 19 different collagens of *Caenorhabditis elegans* (Supplementary Table S2.7). The collagens of *H. contortus* are not closely related to those shown to be crucial for cuticle synthesis in *Caenorhabditis elegans* (i.e., are not DPY-2, -3, -7, -8, -10 and -13) (Page and Johnstone, 2007), indicating significant differences in cuticle synthesis between this free-living nematode and *H. contortus*. The marked growth of *H. contortus* following its moults may be explained by cuticle extension and expansion, and previous work on *H. contortus* has shown that there is a marked increase in the thickness of both the basal and median layers of the cuticle when the L4 (0.089-0.13 μm) develops to the adult stage (0.28-0.41 μm) (Veglia, 1917). Thus, it is likely that the relatively high numbers of collagens and cuticular proteins relate to rapid growth and developmental transitions in *H. contortus* throughout its life cycle.

Parasitic worm proteins that belong to the cysteine-rich secretory proteins, antigen 5 and pathogenesis-related 1 (CAP) superfamily (Gibbs et al., 2008) are recognised to play

key roles in the infection process, in growth and development and/or modulation of immune responses in host animals (Hewitson et al., 2015). Here, we detected 21 SCP/TAPS family proteins, 19 of which were detected in the L4 and adult stages, but not the free-living stages of *H. contortus* (see Supplementary Table S2.8). We propose that that the 19 SCP/TAPS proteins in the parasitic stages play central roles in development, reproductive processes and/or the parasite-host interplay in the host animal, and that the two others (i.e., HCON_10053s and HCON_11608s) have roles in the development of eggs or free-living larval stages. These hypotheses need to be tested in extensive experimental investigations.

A previous genomic study predicted genes encoding 84 SCP/TAPS proteins (22 double and 62 single SCP-like domain molecules) in *H. contortus*, 43 of which were specifically transcribed in both of these stages (Schwarz et al., 2013). The original number of predicted SCP/TAP proteins ($n = 84$) was likely inflated compared with the 21 proteins detected in the present study due to the fragmentation typical of draft genomes assembled from short DNA sequence read data (Korhonen et al., 2016). Sequences of these 21 matched those in the set of 45 predicted from transcriptomic data sets from a previous study (Mohandas et al., 2015). The discrepancy in number between this and the previous study can be explained, to some extent, by detectable proteins being present exclusively in the soma of the worm, or simply by an inability of the present quantitative proteomic approach to detect tiny amounts of proteins in a complex suspension of molecules from the parasite (Schubert et al., 2017). Further work is required to estimate the number of proteins present also in excretory/secretory (ES) products from all stages of *H. contortus.* By comparing the 21 detected proteins with those inferred from transcriptomic data (Mohandas et al., 2015), ~23 would be expected to be in ES products, provided that they are detectable using current proteomic tools (analytical sensitivity of 1×10^{-15} g) (Yates et al., 2009), but further work is required to confirm this proposal. The previous discovery of only two SCP/TAPS proteins (i.e., Hc24 - single SCP-like domain, and Hc40 - double SCP-like domain) in ES products from adult *H. contortus* (see Yatsuda et al., 2003) likely related to the limited level of sensitivity of the two-dimensional, gel-based proteomic method used and the absence of a genome at the time of investigation.

Besides SCP/TAPS proteins, proteins of the C-lectin family were abundant in different developmental stages (cf. Supplementary Table S2.9). C-type lectins are carbohydrate-

binding proteins (McGreal et al., 2004; Mulvenna et al., 2009) and likely play roles in parasitism, immune evasion (Harcus et al., 2009), defence against microbes (Mallo et al., 2002) and/or and fertilisation (Brown et al., 2007). Molecular evidence indicates that the C-type lectin CLEC-50 has an 'inducible' antibacterial effect in the free-living nematode *Caenorhabditis elegans* (Mallo et al., 2002). In parasitic nematodes, similar C-type lectin homologues have been shown to be expressed primarily in the adult stages of the strongylid nematodes *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, suggesting an anti-bacterial role (Harcus et al., 2009). However, at least 270 C-type lectins have been inferred for *Caenorhabditis elegans* (Schulenburg et al., 2008), whereas eight C-type lectins were detected here in the proteome of *H. contortus*, with 46 predicted previously from the draft genome of this worm (Schwarz et al., 2013); this difference in number likely relates to the fragmentation that is characteristic of draft genomes (Korhonen et al., 2016).

Given that most C-type lectins are expressed in a pathogen-specific manner in *Caenorhabditis elegans* (i.e., one particular C-type lectin expressed to defend against one particular pathogen species) (Schulenburg et al., 2008), it is likely that the regulation of these lectin in defence against bacterial infections is distinct between *Caenorhabditis elegans* and *H. contortus*. One C-type lectin in *H. contortus* might respond to multiple pathogens species, although this worm (at the L4 and adult stages) lives in what could be considered as a sterile environment (pH 1.0 in the abomasum). It would be interesting to establish whether C-type lectins are upregulated in this parasitic nematode in response to particular pathogens or microbes. It is also possible that some C-type lectins play a role in immune evasion, as it is known that TES-70 in *Toxocara canis* and *As*-CTL-1 in *Ascaris suum* exhibit sequence homology (28-31%) to host immune cell receptors (e.g., CD23 and macrophage mannose receptor) (Loukas et al., 1999; Yoshida et al., 2012), and they have been reported to bind mammalian carbohydrates in a calcium-dependent manner (Loukas et al., 2000). This information suggests a role for C-type lectins in immune evasion by parasitic nematodes either via inhibiting the migration of host cells or by binding to, or masking worm carbohydrates from/on host immune cells. The abundant expression of C-type lectins in parasitic stages of *H. contortus* (see Supplementary Table S2.9) suggests that these proteins could be involved in immune evasion or avoidance in the host animal. Interestingly, four C-type lectins, HCON_2564s,

HCON_6854s, HCON_8701s and HCON_8702s, were present at high levels in the egg stage of *H. contortus* (see Supplementary Table S2.9). A previous study of related strongylid, *Ancylostoma ceylanicum* (hookworm), showed that a C-type lectin, designated *Ace*-CTL-1, expressed in the sperm and testes of adult males, and the spermatheca and developing embryos within adult females, is involved in reproductive and developmental processes of this hookworm (Brown et al., 2007). This information stimulates further explorations of the functional roles of HCON_2564s, HCON_6854s, HCON_8701s and HCON_8702s in *H. contortus.*

In conclusion, here we report the somatic proteome of the barber's pole worm, one of the most important pathogens of ruminants world-wide. Using a high throughput LC-MS/MS-based approach, we confidently quantified a total of 2487 proteins in key developmental stages (i.e., egg, L3, L4 and adults) and observed marked proteomic alterations during the developmental transition from the free-living to the parasitic stages, particularly for molecules inferred to play essential roles in host invasion, development and survival (i.e., nutrition metabolism, cuticle synthesis/turnover, parasite-host communication/interaction, blood feeding and detoxification), suggesting a fine biological balance between the parasite and the host animal. The present results now complement and further enhance the value of transcriptomic and genomic data sets, will enable new insights into the biology of this highly significant parasitic nematode and should underpin work toward new anti-parasite interventions in this post-genomics era. From a technical perspective, this study indicates the major advantages of a high throughput LC-MS/MS-based approach to support systems biology explorations of parasitic nematodes and encourages proteomic studies of other socio-economically important parasitic nematodes.

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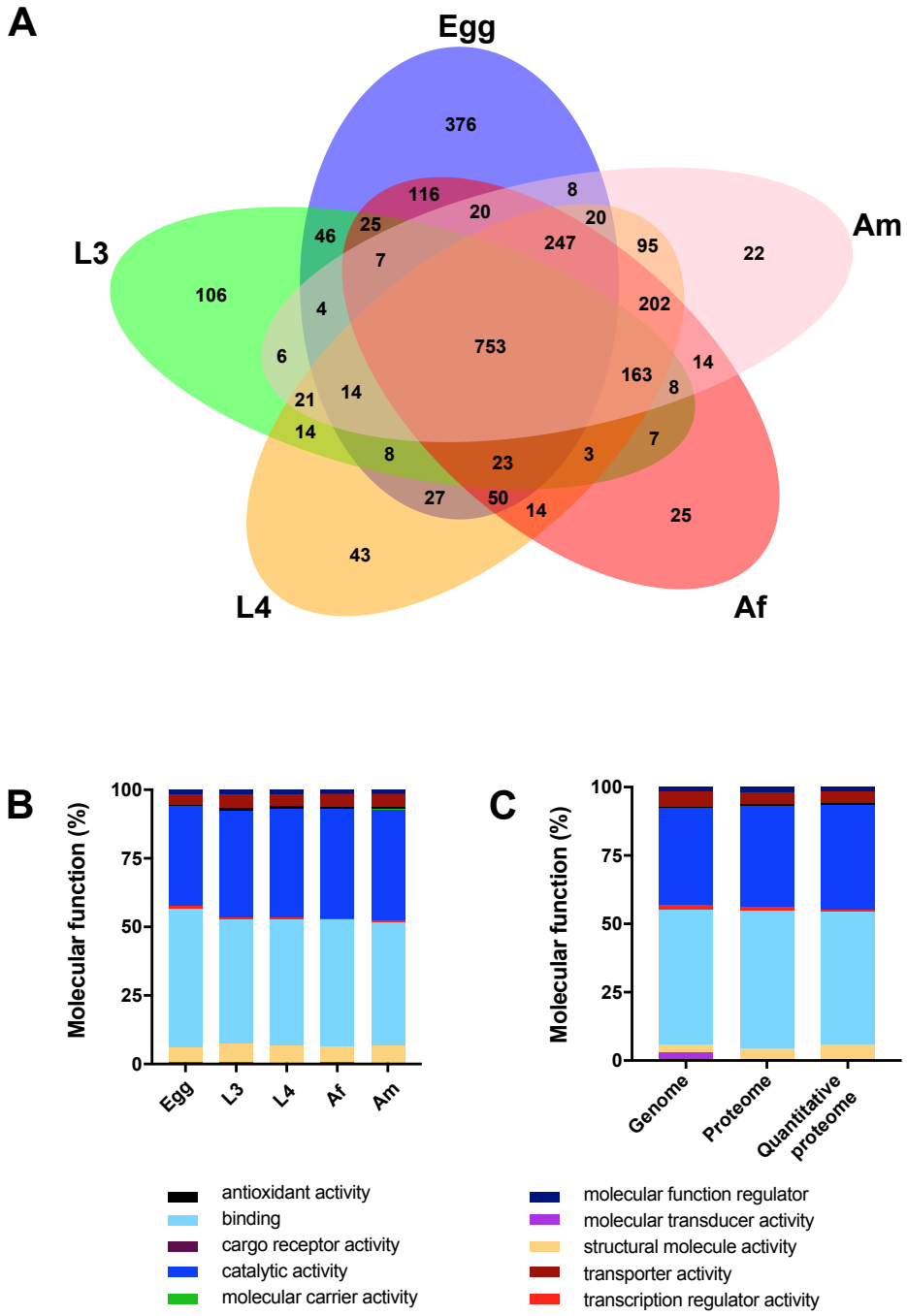


Fig. 2.1. *Haemonchus contortus* proteins and molecular fucntions. (A) Venn diagram showing the numbers of quantified proteins unique to or shared by different developmental stages of *H. contortus* [egg, third-stage (L3), fourth-stage (L4) larvae, female (Af) and male adults (Am)]. (B) The distribution of the molecular functions (Gene Ontology (GO) level 2) of proteins quantified in each stage. Distribution was expressed as a percentage of the total number of proteins identified in a particular stage, to allow quantitative comparisons among different developmental stages of *H. contortus*. (C) The distribution of the molecular functions (GO level 2) of predicted or identified proteins. Distribution was expressed as a percentage of the total number of proteins, to allow quantitative comparisons on the genome, qualitative, and quantitative proteome levels.

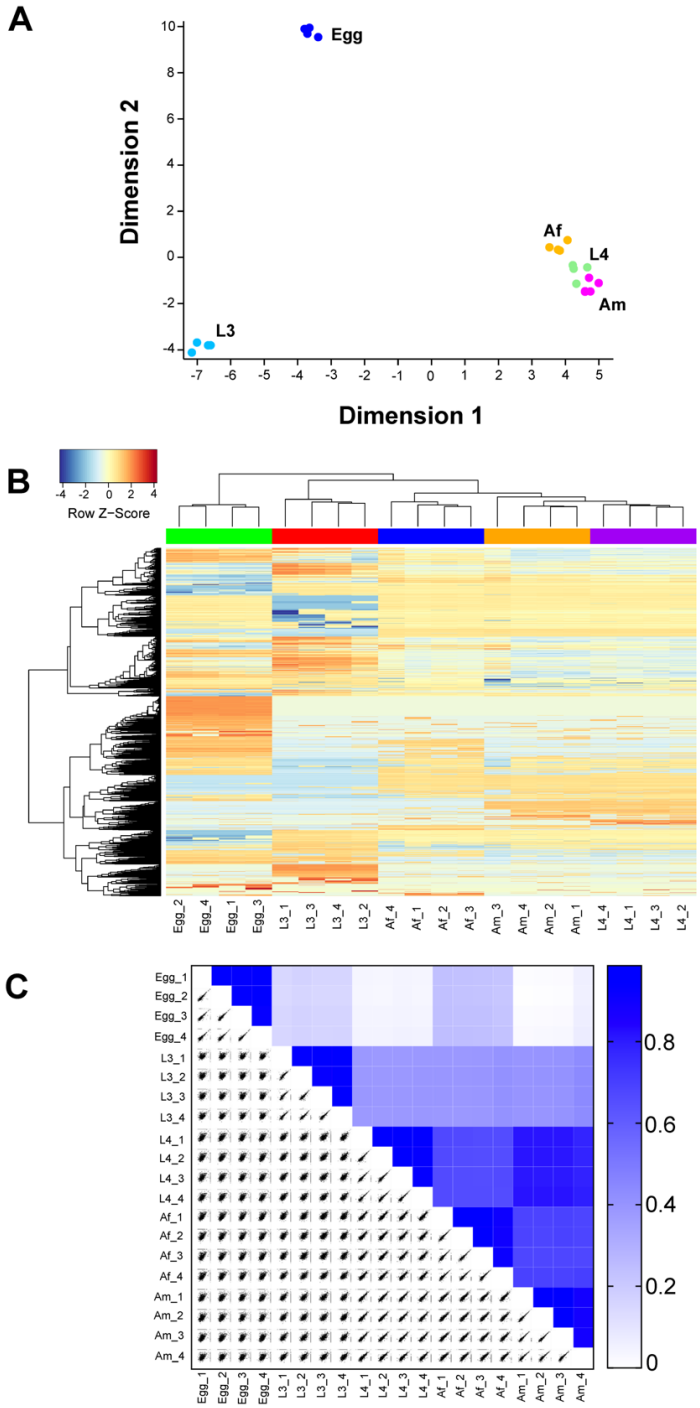


Fig. 2.2. (A) Principal component analysis (PCA) of the somatic proteome of *Haemonchus contortus* representing eggs, third-stage (L3), fourth-stage (L4) larvae, female (Af) and male (Am) adults. (B) Heatmap displaying the expression profiles for these distinct developmental stages/sexes. Normalised protein abundance is shown as a blue to red scale, depicting low to high protein abundance. (C) Multi-scatter plots showing the correlation between biological replicates of proteomic changes in developmental stages upon pairwise comparison. Dark blue represents a high Pearson's correlation between samples. Each stage was represented by biological quadruplicates.

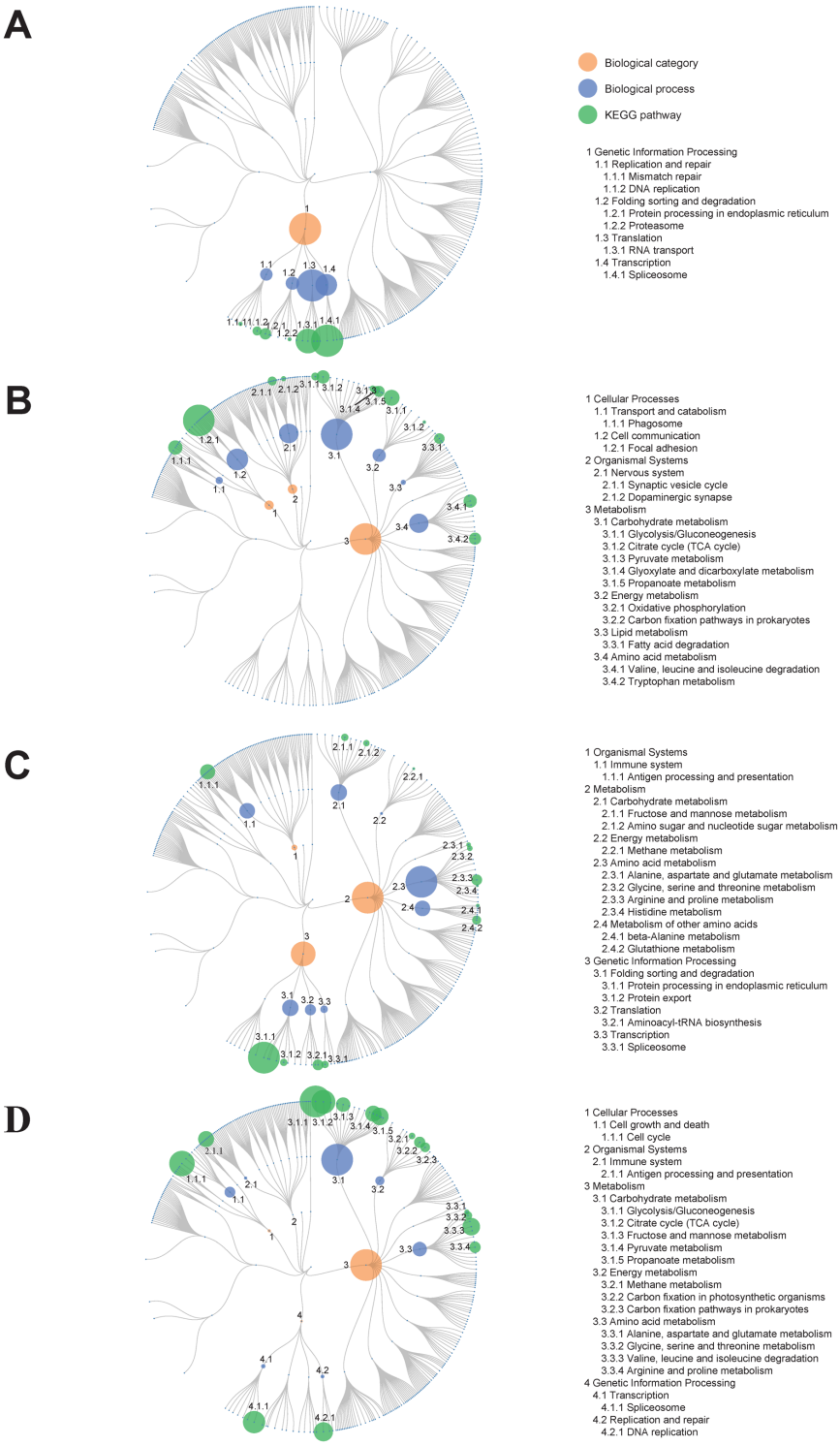


Fig. 2.3. Enriched biological processes and associated pathways (KEGG) of differentially expressed proteins in (A) Egg, (B) L3, (C) L4, (D) Af stages of *Haemonchus contortus*. Enriched annotations of highly expressed proteins [fold change (FC) of > 2; false discovery rate (FDR) of < 0.05] are listed. Dot sizes indicate counts of significantly highly expressed proteins (see also Supplementary Table S2.5).

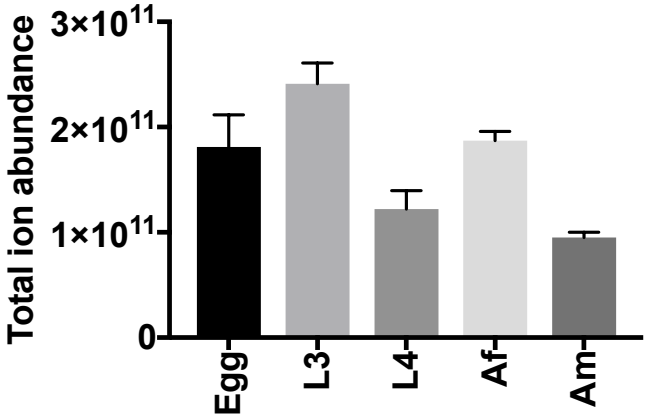


Fig. 2.4. Comparison of total ion abundance of lipids among different developmental stages/sexes of *Haemonchus contortus* [egg, third-stage (L3), fourth-stage (L4) larvae, female (Af) and male adults (Am)]. Statistical analysis was performed by ANOVA (** $P < 0.01$). Error bars indicate ± standard deviation (SD) of the mean (four biological replicates).

Supplementary Table S2.1. Proteins identified for constructing the DIA ion library from key different developmental stages of *Haemonchus contortus* (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S2.2. The full list of proteins quantified in the *Haemonchus contortus* proteome of key different developmental stages (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S2.3. The number of quantified proteins involved in the molecular function (levels 2 and 3) in each stage of *Haemonchus contortus* (i.e., eggs; third-stage and fourth-stage larvae; female and male adults), according to Gene Ontology (GO).

Supplementary Table S2.4. The numbers of predicted, identified and quantified proteins involved in the molecular functions (level 2) according to the Gene Ontology (GO).

Supplementary Table S2.5. Enriched biological categories, biological processes and KEGG pathways of differentially expressed proteins in different developmental stages of *Haemonchus contortus* (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S2.6. Lipid species quantified in different developmental stages/sexes of *Haemonchus contortus* and employed for the analysis of total lipid abundance.

Supplementary Table S2.7. The full list of quantified collagens and cuticular proteins in the proteome of *Haemonchus contortus* representing key different developmental stages (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S2.8. The full list of quantified SCP/TAPS family proteins in the proteome of *Haemonchus contortus* representing key different developmental stages (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S2.9. The full list of quantified C-type lectins in the proteome of *Haemonchus contortus* representing key different developmental stages (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

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Chapter 3. High throughput LC-MS/MS-based proteomic analysis of excretory-secretory products from short-term in vitro culture of *Haemonchus contortus*

Abstract

Parasitic nematodes of humans, animals and plants have a major, adverse impact on global health and agricultural production worldwide. To cope with the immediate environment and the immune attack from the host, excretory-secretory (ES) proteins are released by nematodes to orchestrate or regulate parasite-host interactions. In the present study, we characterised the ES products from a short-term in vitro culture (12 h) of *Haemonchus contortus* (as the most important parasitic nematodes of livestock animals worldwide) using a high throughput tandem mass-spectrometry, underpinned by the most recent genomic dataset. In total, 878 unique proteins from key developmental stages/sexes (third-stage and fourth-stage larvae, and female and male adults), cultured under in vitro condition, were identified and quantified with high confidence. Bioinformatic analyses showed noteworthy ES protein alterations during the transition from the free-living to the parasitic phase, especially for ES proteins which are likely involved in nutrient digestion and acquisition as well as parasite-host interactions, such as proteolytic cascade-related peptidases, glycoside hydrolases, C-type lectins, transthyretin-like proteins and SCP/TAPS (Sperm-coating protein/Tpx/Antigen 5/Pathogenesis related-1/Sc7) proteins. Our findings provide an avenue to better explore interactive processes between the host and this highly significant parasitic nematode, to underpin the search for novel drug and vaccine targets.

3.1. Introduction

Parasitic nematodes cause diseases that have an adverse impact on human health and agricultural production (Roeber et al., 2013; Charlier et al., 2014; Jourdan et al., 2017). For instance, the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*) and hookworms (*Necator americanus*, *Ancylostoma duodenale* and *Ancylostoma ceylanicum*) are soil-transmitted helminths that infect ~1.5 billion people globally and affect some of the most disadvantaged communities (http://www.who.int/mediacentre/-factsheets/fs366/en/). In an agricultural context, strongylid nematodes, including the barber's pole worm (*Haemonchus contortus*), infect hundreds of millions of livestock animals (e.g., sheep and goats) worldwide, and cause deaths and production losses estimated at $3.3 billions per annum (Evans and Chapple, 2002). The control of parasitic nematodes relies heavily on chemotherapeutic treatment. However, the excessive use of chemical drugs has led to widespread anthelmintic resistance in numerous worm species (Kaplan and Vidyashankar, 2012; Martin et al., 2016). This situation has stimulated the discovery and development of novel drugs and vaccines (Loukas et al., 2011; Geary et al., 2015).

The excretory-secretory (ES) molecules produced by parasitic nematodes and presented at the nematode-host interface are proposed to be useful targets for drugs and/or vaccines (Hewitson et al., 2008; Hewitson et al., 2009). These molecules, including proteins, carbohydrates and lipids, are actively released from the alimentary tract of the worm, through particular excretory glands or originate from the cuticular surface, and appear to maintain a physiological and/or immunological relationship with the host (Maizels, 2009). Characterising these worm components and understanding their functions within the host animal should provide an avenue to better explore interactive processes between the nematode and host, and could pave way to discovering new strategies to combat worms.

Due to technical limitations, previous studies of nematode secretomes were carried out mainly to investigate the chemical composition, ultrastructure and/or immunological roles of selected molecules (Kennedy and Qureshi, 1986; Kennedy et al., 1989; Schallig et al., 1994; Vervelde et al., 2002). However, in recent years, the advent of mass spectrometric (Aebersold and Mann, 2016) and sequencing-bioinformatic (Leipzig,

2017) technologies have enabled large-scale investigations. To date, the genomes of > 70 species of parasitic nematodes have been sequenced and annotated (International Helminth Genomes Consortium, 2019). Despite this expansion, the number of proteomic studies is small. Published proteomic studies of the secretomes of parasitic nematodes, including *Ascaris suum* (see Wang et al., 2013; Chehayeb et al., 2014), *Brugia malayi* (see Hewitson et al., 2008; Moreno and Geary, 2008; Bennuru et al., 2009), *Bursaphelenchus xylophilus* (see Shinya et al., 2013), *Dirofilaria immitis* (see Geary et al., 2012) and *Toxocara canis* (see Sperotto et al., 2016), have been substantially facilitated through the use of genomic and trancriptomic datasets. Draft genomes of *H. contortus*, as one of the most socio-economically important nematodes of livestock animals, were published in 2013 (Laing et al., 2013; Schwarz et al., 2013) and updated more recently (Doyle et al., 2018). Although some previous studies (Schallig et al., 1994; Vervelde et al., 2002) have showed that the injection of ES molecules from *H. contortus* or their purified fractions are able to protect sheep by up to 90% against experimental challenge infection with *H. contortus*, surprisingly, there has been no profound characterisation of the proteomic secretome of *H. contortus*, with the exception of some early studies using traditional techniques, available at the time (Gamble and Mansfield, 1996; Yatsuda et al., 2003). The aim of the present study was to comprehensively characterise the secretome of key developmental stages of *H. contortus* using tandem mass-spectrometry, underpinned by the most recent genomic data set for this nematode (Doyle et al., 2018). We explored aspects of this secretome to start to understand parasite-host and/or parasite-parasite interactions and to provide an improved basis for the discovery of novel therapeutic and/or vaccine targets.

3.2. Materials and methods

3.2.1. Parasite procurement

Haemonchus contortus was produced in Merino lambs (6 months of age; Victoria, Australia), maintained under helminth-free conditions in facilities in the University of Melbourne. Animal ethics approval (no. 1714374) was granted by the University of Melbourne, which follows Part 3 of the Prevention of Cruelty to Animals Act 1986 and

Part 4 of the Prevention of Cruelty to Animals Regulations 2008 of the State of Victoria as well as the Australian Code for the Care and Use of Animals for Scientific Purposes 1969 ("the Australian Code").

Sheep were inoculated intra-ruminally (via oral intubation) with 7000 infective (pre-parasitic) L3s of *H. contortus*. One month following inoculation, eggs were isolated from the faeces from infected sheep using a sucrose flotation procedure (Mes et al., 2007). Other developmental stages of *H. contortus* - i.e., third-stage (L3) and fourth-stage (L4) larvae; female (Af) and male (Am) adults - were produced as described previously (Schwarz et al., 2013). In brief, L3s were produced by coproculture (Nikolaou et al., 2002), and L4s and adult worms (sexes separated) were collected from the abomasa of infected sheep 9 and 28 days following inoculation of sheep with L3s, respectively. Four biological replicates of each developmental stage/sex were used in each experiment.

3.2.2. Preparation of excretory/secretory products

All of the individual life stages of *H. contortus* collected were extensively washed in physiological saline (37 °C) and then RPMI 1640 medium (37 °C, pH 7.2) with 10 mM L-glutamine (BioWhittaker; cat no. 12-702F; Lonza). Subsequently, worms of each developmental stage were suspended in 200 ml culture fluid [RPMI 1640 with 10 mM L-glutamine (BioWhittaker; cat. no. 12-702F; Lonza) containing final concentrations of 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin (fungizone, antibiotic-antimycotic; cat. no. 15240-062; Gibco, USA)] in individual tissue culture flasks (175 cm^2, vented cap; DB Falcon) at differing concentrations (30,000 L3, 100 L4, 30 Af and 2 Am worms per 10 ml), and then incubated at 37 °C in 10% v/v CO_2. The viability of the worms was assessed every 3 h, and the culture medium was collected after 12 h. To eliminate any host tissue contamination, culture medium was discarded after first 3 h of culture and replaced with fresh medium (37 °C, 10% v/v CO_2). Following maintenance culture for 12 h, the medium was differentially centrifuged to remove intact worms (480 ×*g* for 5 min) and cell debris (2,000 ×*g* for 10 min), and then concentrated to ~1 ml by centrifugal ultrafiltration (molecular weight cut-off: 3,000 Da; cat. no. UFC900324; Amicon, Millipore) at 4 °C. Protein concentrations were measured using a standard bicinchoninic acid (BCA) assay (cat. no. 23227; Thermo Fisher Scientific,

USA).

3.2.3. In-solution digestion of proteins, and LC-MS/MS analysis

The analysis of the ES proteome of *H. contortus* was conducted using in-solution digestion protocol, as described previously (Ang et al., 2011). Samples containing ES proteins (50 µg) from either the L3, L4, Af or Am stage were reduced with 10 mM tris (2-carboxyethyl) phosphine (TCEP), alkylated with 55 mM iodoacetamide, followed by a double-digestion with Lys-C/trypsin mix (Promega, USA) at 37 °C for 16 h (4 h for Lys-C digestion and 12 h for trypsin digestion). The tryptic samples were acidified with 1.0% (v/v) formic acid, purified using Oasis HLB cartridges (Waters, USA) and spiked with the HRM calibration peptides (Biognosys, Switzerland). Each protein sample was separated into eight fractions using the high pH reversed-phase peptide fractionation kit (Pierce, USA), according to the manufacturer's protocol. All fractions were freeze-dried prior to re-suspension in aqueous 2% w/v acetonitrile and 0.05% trifluoroacetic acid (TFA) w/v before LC-MS/MS analysis.

Tryptic peptides were analysed using Q Extractive Plus Orbitrap mass spectrometers (Thermo Fisher, USA). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 µm × 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 µm × 50 cm). The tryptic peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 2% v/v CH_3CN containing 0.05% v/v trifluoroacetic acid for 6 min, applied before the enrichment column was switched in-line with the analytical column. The eluents were 0.1% v/v formic acid (solvent A) in H_2O and 100% v/v CH_3CN in 0.1% v/v formic acid (solvent B), both supplemented with 5% dimethyl sulfoxide. The gradient was at 300 nl/min from (i) 0-6 min, 3% B; (ii) 6-95 min, 3-20% B; (iii) 95-105 min, 20-40% B; (iv) 105-110 min, 40-80% B; (v) 110-115 min, 80-80% B; (vi) 115-117 min, 85-3%, and equilibrated at 3% B for 10 min before injecting the next sample. The Q Extractive Plus mass spectrometer was operated in the data-dependent mode, whereby full MS1 spectra were acquired in a positive mode, 70,000 resolution, AGC target of $3e^6$ and maximum IT time of 50 ms. Fifteen of the most intense peptide ions with charge states of ≥ 2 and intensity thresholds of ≥ $1.7e^4$ were isolated for MS/MS. The isolation window was set at 1.2 m/z, and

precursors were fragmented using higher energy C-trap dissociation (HCD) at a normalised collision energy of 30, a resolution of 17,500, an AGC target of $1e^5$ and a maximum IT time of 100 ms. Dynamic exclusion was set at 30 sec. The Q Extractive Plus mass spectrometer was operated in positive-ionisation mode, with the spray voltage set at 1.9 kV and the source temperature at 275 °C.

3.2.4. Protein identification and quantification

The proteome predicted for *H. contortus* (see Doyle et al., 2018) was annotated using NCBI non-redundant (NR) protein database (Pruitt et al., 2012). Raw data were processed using MaxQuant (Tyanova et al., 2016a). Search parameters were: a precursor tolerance of 20 ppm, MS/MS tolerance of 0.05 Da, fixed modifications of carbamidomethylation of cysteine (+57 Da) and methionine oxidation (+16 Da). Peptides were accepted based on a false discovery rate (FDR) of < 0.01 at both the peptide and protein levels. Proteins were quantified using the LFQ value from MaxQuant employing default settings. For stage-specific identification and relative quantification comparisons, only proteins identified with ≥ 2 peptides and detected in ≥ 3 biological replicates of at least one developmental stage/sex were accepted. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD012503.

3.2.5. Bioinformatic analyses of data sets

In addition to the annotations for the proteome of *H. contortus* (see Doyle et al., 2018), proteins were classified by their Gene Ontology (GO) using the program InterProScan v5.15.54 (Mitchell et al., 2015). Principal component analysis (PCA), hierarchical cluster analysis (HCA) and multi-scatter plots were conducted using Perseus software (v.1.6.1.1) (Tyanova et al., 2016b; Tyanova and Cox, 2018). The statistical significance of correlation analyses was set at $P < 0.05$. The protein sequences were examined for the presence of signal peptides and transmembrane regions using programs SignalP 3.0 and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Subcellular localisation was predicted using the program SecretomeP 2.0 (Bendtsen et al., 2005).

Sequences were aligned using the program MUSCLE (Edgar, 2004) and trimmed manually. Bayesian inference (BI) trees were constructed using MrBayes v3.2.3 (Ronquist and Huelsenbeck, 2003); likelihood parameters set in the BI analysis of sequence data were based on the Akaike Information Criteria test in ProtTest v3 (Darriba et al., 2011). Aligned amino acid sequence data were subjected to analysis using the 'mixed' rate matrix setting. For each tree, posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains, with trees being saved every 100th generation. Burn-in was set at 0.25, and a majority rule consensus tree was generated. The tree was viewed using the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and modified in Inkscape (http://www.inkscape.org/en/).

3.3. Results

In total, 878 proteins were identified and quantified with high confidence using stringent criteria (i.e., represented by ≥ 2 peptides in ≥ 3 biological replicates in at least one developmental stage/sex) in the ES products of all key developmental stages/sexes (i.e., L3, L4, Af and Am) of *H. contortus*. More ES proteins were identified in L4 (*n* = 535) and Af (*n* = 544) than in L3 (*n* = 311) and Am (*n* = 484). In silico analyses predicted that 512 (58.3%) of the 878 ES proteins were secreted via a classical or non-classical pathway, and included peptidases (*n* = 115), SCP/TAPS (Sperm-coating protein/Tpx/Antigen 5/Pathogenesis related-1/Sc7) proteins (*n* = 52) and transthyretin-like proteins (*n* = 28).

The full list of ES proteins identified herein is given in Supplementary Table S3.1, and the numbers of proteins in the distinct stages/sexes of *H. contortus* are displayed in a Venn diagram (Fig. 3.1A). More than half of ES proteins (*n* = 516, 58.8%) were shared by at least two developmental stages, 119 (13.5%) of which were common among all stages/sexes studied. Notably, a relatively large number (*n* = 224) of ES proteins identified were exclusively shared by parasitic stages (i.e., L4, Af and Am), representing 41.9%, 41.2% and 46.3% of the sub-totals, respectively. By contrast, only small numbers of ES proteins detected in the free-living L3 stage were also identified exclusively in L4 (*n* = 23, 2.6%), Af (*n* = 3, 0.3%) or Am (*n* = 5, 0.6%). Apart from the latter proteins, 143

(16.3%), 85 (9.7%), 84 (9.6%) and 50 (5.7%) proteins appeared to be specific to L3, L4, Af and Am, respectively. In total, 567 ES proteins were inferred to be specific to L4, Af and/or Am (Supplementary Table S3.1). Apart from the peptidases (*n* = 86), SCP/TAPS proteins (*n* = 37), C-type lectins (*n* = 17), transthyretin-like proteins (*n* = 14) and glycoside hydrolases (*n* = 9) were also conspicuous in the parasitic stages studied (Fig. 3.1A).

Overall, most ES proteins identified in L3 (81.4%), L4 (85.8%), Af (83.8%) and Am (82.0%) were homologous (e-value cut-off: 10^{-5}) to known proteins (Supplementary Table S3.1). The distribution of the molecular functions (level 2) of the proteins in each stage, according to information in the GO database, is shown in Fig. 3.1B. The distribution of GO terms indicated that catalytic activity (GO: 0003824; 46.3-52.3%) and binding (GO: 0005488; 37.7-41.3%) were the two predominant molecular functional categories for the developmental stages/sexes investigated. Each of these categories represented > 70 annotated proteins, with proteins associated with various activities/processes, such as antioxidant activity, molecular transducer regulator and structural molecule activity, being less represented (Supplementary Table S3.2). A more detailed appraisal (level 3) of the two main molecular functional categories revealed that catalytic activity via hydrolases predominated, while other proteins were inferred to mainly be involved in the binding of compounds, ions, proteins and/or small molecules (Supplementary Table S3.2). Nevertheless, there was no stage/sex-associated difference in the proportions (percentages) of proteins classified in each functional category (Fig. 3.1B; Supplementary Table S3.2), with similar results obtained for the distribution of the Biological Processes (level 2) and Cell Components (level 2) (Supplementary Tables S3.3 and S3.4).

Multi-scatter plot analysis of the secretome of *H. contortus* showed that difference in the identity and quantity of ES proteins among L3, L4, Af and Am was greater than variation within a particular stage (i.e., among the four replicates) (Fig. 3.2A). Subsequent PCA showed that the four replicates representing individual developmental stages/sexes of *H. contortus* clustered together, with a clear distinction in the clustering among them (Fig. 3.2B). The hierarchical clustering also showed a clear division of the proteomic data set into four distinct groups, corresponding to the four different developmental stages (Fig. 3.2C).

Functionally relevant proteins, including peptidases and transthyretin-like proteins, of the secretome of *H. contortus* were analysed further. Distinct proteolytic cascades were inferred to be represented by four classes of peptidases [i.e., aspartic (n = 30), cysteine (n = 37), metallo- (n = 31) and exo- (n = 7) peptidases] (Fig. 3.3). In addition, relative quantification showed that most peptidases (n = 96) related to the parasitic stages (i.e., L4, Af and Am), whereas a limited number (n = 25) were identified in the free-living L3 stage (Fig. 3.3). A phylogenetic analysis showed that transthyretin-like proteins representing all developmental stages/sexes studied clustered together, whereas those excreted/secreted exclusively by L4, Af and Am tended to group together, with the exception of two molecules (i.e., HCON_00137540 and HCON_0014862) (Fig. 3.4).

3.4. Discussion

Based on current knowledge, there are two main modes of protein secretion, i.e., classical secretion through secretory portals of the cell plasma membrane (Lee et al., 2012), and non-classical secretion via various non-classical pathways, such as the direct translocation of proteins across the plasma membrane, blebbing, lysosomal secretion and release via exosomes derived from multi-vesicular bodies (reviewed in Nickel, 2003). In the present study, 54.8% (n = 481) of ES proteins identified were predicted to be secreted via classical pathways and 57.1% (n = 501) via non-classical ones. These percentages are consistent with those established in previous ES proteomic investigations of other parasitic nematodes, such as *Ascaris suum* and *Brugia malayi* (see Bennuru et al., 2009; Wang et al., 2013).

Most previous studies yielded parasite ES material by culturing nematodes in vitro and collecting the culture medium either daily or at the end of a culture period of up to 3-5 days. Although most studies verified worm viability and integrity to avoid contamination by components derived from dead or dying worms, the presence of some typical intracellular proteins, such as histones (Guillou et al., 2007; Liu et al., 2009; Mulvenna et al., 2009; Virginio et al., 2012; Wang et al., 2013), suggested leakage of intracellular contents following cellular damage into the culture medium. In the present study, we cultured *H. contortus* stages for 12 h to avoid contamination of medium by intracellular proteins. Sound results were achieved in that we identified a large number of ES proteins

(*n* = 878; Supplementary Table S3.1) without evidence of intracellular proteins typically detected in longer term maintenance cultures (Wang et al., 2013), and we showed that many of the proteins identified represent known, key molecules involved in host-parasite interactions (including physiological, immune, hormonal and adaptive responses or processes) (cf. Hoste, 2001; Maizels and McSorley, 2016). From a methodological perspective, the short-term (12 h) in vitro culture approach, combined with the use of a high analytical sensitivity of 1×10^{-15} g (Yates et al., 2009) of mass spectrometry, should be readily applicable to any parasitic nematode. We do acknowledge, however, that in vitro conditions employed here are distinct from those within the abomasum (stomach) of the host animal (ruminant) and, thus, not entirely physiological, and that pH, antibiotic/antimycotic supplements in the culture medium used and worm density might alter the in vitro-secretome of *H contortus* compared with that expressed and released in vivo within the host. While challenging, future work should attempt to compare the secretomes of the parasitic (L4 and adult) stages and both sexes of *H. contortus* produced in vitro with those raised in vivo. This might be achievable using filtration membrane diffusion chambers containing larvae anchored to the abomasum wall, followed by proteomic analysis. However, a suitable and reproducible approach would need to be established.

From a biological perspective, the success of parasites is critically dependent on their unique abilities to adapt to their immediate host environment (Maizels and McSorley, 2016). In the life cycle of *H. contortus*, the free-living L3s are ingested orally by the ruminant host, after which exsheathed larvae invade and establish in the stomach of the host to commence the parasitic phase (i.e., L4 and adults) of the life cycle and feed on blood. During the transition from the free-living to the parasitic phase, nematodes likely orchestrate their production of ES components to meet the demands for rapid growth, development and survival (e.g., through host cross-talk and immunomodulation). Intriguingly, many ES proteins identified in this study are not only proposed essential molecules, namely, peptidases, glycoside hydrolases, C-type lectins, transthyretin-like proteins and SCP/TAPS proteins, but also many of them appear to be specific to a particular developmental stage or sex.

Limited by the current biotechnology and biochemistry tools, it is still a challenge to characterise the ES products released/secreted inside the host in a real time with a high

throughput manner. At the current stage, the prerequisite to be able to enrich and characterise the secretome of parasitic nematodes is maintain the parasites under an artificial environment.

However, parasites as any other organisms have the ability to adapt to the immediate environment (Yates and Ruse, 2009), and their adaptations are not only limited to the host immune response, but also include the physiological and non-physiological factors in the immediate environment. In the present study, although we minimised the non-physiologically relevant maintenance time and many of identified molecules were proved to be the key players in the host-parasite in previous studies, we should still keep in mind that the pH, the antibiotics and antimycotics of the culture medium as well as the worm density of culture medium could cause the dynamic change in the secretome of parasitic nematodes. To optimise the in vitro culture system, it is worth to define/understand the impact of these non-physiological factors on the secretome of parasitic worm in the future study.

3.4.1. Proteolytic enzymes

In terms of the feeding on and digestion of host proteins, a series of proteolytic cascade-related peptidases (n = 105) were predominantly represented in the secretome of *H. contortus* (Supplementary Table S3.1; Fig. 3.3). These molecules are the major haemoglobin degradation and anticoagulation enzymes, and are presumed to be essential for the growth, development and survival of this and other haematophagous parasitic nematodes [e.g., *Ancylostoma* and *Necator americanus*; cf. (Williamson et al., 2003; Perner et al., 2019)]. It has been postulated that a series of peptidases is required to degrade host haemoglobin in the gut of blood-feeding nematodes (Williamson et al., 2003): host haemoglobin is initially hydrolysed into long peptides by aspartic peptidases, substantially degraded into smaller peptides by cysteine peptidases and then by metallopeptidases, eventually being cleaved by exopeptidases into absorbable nutrients (i.e., small peptides or free amino acids) (illustrated in Fig. 3.3). Interestingly, a series of these molecules representing four distinct classes of peptidases [i.e., aspartic (n = 30), cysteine (n = 37), metallo- (n = 31) and exo- (n = 7) proteases] were identified among the ES proteins studied here (Fig. 3.3). Additionally, in contrast to the free-living L3 stage,

most of these peptidases (76.2%) predominated in the parasitic L4, Af and Am stages (Fig. 3.3), suggesting heavy demands on the catabolism of haemoglobin once a blood-feeding nematode establishes in the gastrointestinal tract of the host (Ranjit et al., 2009). Due to their essential roles in digestion/degradation and detoxification processes in blood-feeding worms, these peptidases were believed to be drug or vaccine targets (Caffrey et al., 2018). Indeed, some of them (e.g., H11 and H-gal-GP) have been pursued as vaccines (Ekoja and Smith, 2010; Meier et al., 2016). Although there is no direct evidence, it was generally believed that the intestinal lumen of blood-feeding worms is the main site of haemoglobin digestion (Williamson et al., 2003), making it theoretically feasible to block or inhibit the growth and detoxification of these blood-feeding nematodes by drugs (Bouchery et al., 2018). However, due to the potential functional redundancy of peptidase groups, it would be wise to have a better understanding of the physicochemical and biological properties of these proteases before investing major efforts in the discovery and development of drugs or vaccines targeting these proteins (Rebello et al., 2018).

3.4.2. Glycoside hydrolases

Apart from peptidases, the relative abundance of glycoside hydrolases (n = 13) in the secretome of *H. contortus* is noteworthy. Most of the hydrolases identified (76.9%) were predicted to be secreted via a classical or non-classical means. Glycoside hydrolases belong to a large carbohydrate degradation enzyme family, which catalyse the hydrolysis glycoside linkage in complex sugars (Henrissat and Davies, 1997). Such enzymes were usually not reported in previous proteomic analyses of ES or somatic proteins of parasitic nematodes, but we saw an abundance of glycoside hydrolases in the ES products of *Ascaris suum* L3s from the lung and L4 from the intestine of infected pigs *Ascaris suum* (see Wang et al., 2013). Additional bioinformatic and enzymatic analyses showed that these molecules are expressed at high levels and are enzymatically active in the intestine of the adult *Ascaris suum*, suggesting that carbohydrate degradation-related metabolism represents an indispensable component of the energy metabolism once *Ascaris suum* is established in the gut of the host. Interestingly, in accord with results for the secretome of *Ascaris suum* larvae, the present analysis of ES proteins of *H. contortus* reveals a high abundance of these carbohydrate degradation-related enzymes in parasitic stages

(Supplementary Table S3.1). In addition, we also recently identified a number (n = 14) of glycoside hydrolases in somatic tissues of *H. contortus* (Wang et al., 2019). Given the detection of significant numbers of carbohydrate degradation enzymes in both ES products and somatic tissues, it seems reasonable to assume that the degradation of complex sugars contributes to an alternative energy source in this blood-feeding nematode (*H. contortus*). Whether similar energy metabolism processes/mechanisms exist in other haematophagous parasitic nematodes remains to be established.

3.4.3. C-type lectins

The relative abundance of C-type lectins in ES proteins of *H. contortus* studied here is of particular interest. Members of the C-type lectin family are characterised by a conserved carbohydrate-recognition domain (of 115-130 amino acids), which contains multiple calcium ion-binding sites. These molecules are widely distributed and often abundantly expressed in many animals, and have diverse functional roles in the physiology, immunology and apoptosis of cells (McGreal et al., 2004; Mulvenna et al., 2009). C-type lectins have been identified in the secretomes of nematodes such as *Ascaris suum*, *Ancylostoma ceylanicum*, *Brugia malayi*, *Caenorhabditis elegans*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Toxocara canis* (see Bauters et al., 2017). It has been proposed in previous studies that C-type lectins have antibacterial activities and are involved in parasite-host interactions or immune evasion (Bauters et al., 2017). Nonetheless, in *H. contortus*, the actual function of C-type lectins is still unknown. However, in other nematode species, including *Caenorhabditis elegans*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Toxocara canis*, comprehensive studies have showed that C-type lectins are critical for nematode survival (Loukas et al., 2000; Harcus et al., 2009; Pees et al., 2017). C-type lectins, such as CLEC-60, have been shown experimentally to be upregulated in *Caenorhabditis elegans* exposed to bacteria (e.g., *Microbacterium nematophilum*) and are likely involved in the defence against microbes (O'Rourke et al., 2006). In parasitic nematodes, C-type lectin genes were reported to be expressed predominantly in the gastrointestinal-dwelling stages of *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, possibly suggesting an antibacterial role as well (Harcus et al., 2009). Additionally, some C-type lectins might

be involved in immune evasion in the host animal. For instance, some C-type lectins, such as TES-70 in *Toxocara canis* and *As*-CTL-1 in *Ascaris suum*, show sequence homology (~30%) to mammalian C-type lectins, and might thus act as competitors for host immune receptors (such as natural killer, IgE and macrophage/dendritic cell receptors) (Loukas et al., 2000; Yoshida et al., 2012). Besides, the recombinant *Tc*-CTL-1 of *Toxocara canis* has been shown to bind mammalian carbohydrates in a calcium-dependent manner, indicating a role in immune evasion by utilising C-type lectins to mask the worm with host carbohydrates, to avoid pathogen recognition by the host animal (Loukas et al., 2000).

It is interesting that C-type lectins seem to be excreted/secreted prominently in a stage-specific manner in *H. contortus*. Here, at least 17 C-type lectin-domain containing molecules were identified. Three of them (i.e., HCON_00099460, HCON_00118430 and HCON_00162030) were detected exclusively in ES proteins from the free-living L3 stage, whereas the other 14 exclusively represented the parasitic L4, Af and Am stages (Supplementary Table S3.1). The detection of three C-type lectins exclusive to the free-living stage raises the possibility that such larvae utilise these molecules to protect themselves against microbes in the environment, in a manner analogous to the antibacterial defence of homologues found in *Caenorhabditis elegans*. On the other hand, given the sterile environment in the abomasum of the ruminant host (pH = ~1.0), it seems less likely that the parasitic stages of *H. contortus* employ these molecules for the defence against live microbes, although acid resistant bacteria do colonise the abomasum. However, it seems reasonable to propose that all 14 ES proteins in L4, Af and Am stages likely play important, yet undiscovered roles in immune evasion in the host animal.

3.4.4. Transthyretin-like protein

Apart from these molecules, two other groups of proteins (i.e., transthyretin-like proteins and SCP/TAPS proteins) were conspicuously excreted/secreted by *H. contortus*, particularly at the parasitic stages. Interestingly, they have been consistently detected in previous secretome studies of parasitic nematodes of plants, animals and humans (Jacob et al., 2007; Saverwyns et al., 2008; Wilbers et al., 2018); they all belong to nematode-specific protein families whose functions are still mostly enigmatic.

The analysis of ES proteins of parasitic stages of *H. contortus* revealed 15 transthyretin-like proteins (Fig. 3.1; Supplementary Table S3.1). Previous experimental study of *Caenorhabditis elegans* suggested that transthyretin-like proteins might play roles in cell-cell interactions of apoptotic cells and phagocyte receptor (TTR-52; Wang et al., 2010). In the present study, it was interesting to find two distinct patterns of excretion/secretion for the transthyretin-like proteins (i.e., commonly secreted by the stages studied, and specifically produced by parasitic stages of *H. contortus*) (Supplementary Table S3.1). A phylogenetic analysis showed a division of transthyretin-like proteins into two major groups (Fig. 3.4), with one group comprising mainly parasitic transthyretin-like proteins and the other consisting of common transthyretin-like proteins. These findings raise questions regarding the specific roles of these molecules in the free-living and parasitic phases of the life cycle of *H. contortus*. It would be interesting to elucidate the tissue distribution and assess molecular ligand detection of these nematode-specific transthyretin-like proteins, with an emphasis on molecules enriched in the L4, Af and Am stages (such as HCON_00105260, HCON_00137520 and HCON_00137540, with protein abundance changes of > 5-fold).

3.4.5. SCP/TAPS proteins

Members of the SCP/TAPS protein group have been found in every nematode species thus far investigated (Gibbs et al., 2008; Wilbers et al., 2018). Although the functions of most SCP/TAPS proteins are unknown, proposed roles include: (i) nematode survival and/or development within the host; (ii) angiogenic effects; and (iii) neutrophil chemoattractant activity and immune evasion (Hawdon et al., 1996; Tawe et al., 2000; Cantacessi et al., 2009; Cantacessi and Gasser, 2012; Wilbers et al., 2018). Additionally, the potential of SCP/TAPS proteins as vaccine candidates has been proposed for nematode species including *Ancylostoma caninum* (see Bethony et al., 2005), *Cooperia oncophora* (see Gonzalez-Hernandez et al., 2018), *Ostertagia ostertagi* (see Gonzalez-Hernandez et al., 2016) and *Necator americanus* (see Bethony et al., 2008). Various studies have demonstrated that SCP/TAPS are abundantly expressed and/or secreted during the transition from the free-living to the parasitic life stages in nematode species such as *Ancylostoma caninum*, *Cooperia oncophora*, *Heligmosomoides polygyrus*,

Nippostrongylus brasiliensis and *Ostertagia ostertagi* (see Geldhof et al., 2003; Hewitson et al., 2009; Nisbet et al., 2010; Hewitson et al., 2011; Chalmers and Hoffmann, 2012; Sotillo et al., 2014). Limited by the low sensitivity of a two-dimensional gel-based electrophoretic approach and absence of a whole genomic sequence data, a previous proteomic study of ES products from adult *H. contortus* identified only two SCP/TAPS proteins (i.e., Hc24 - single SCP-like domain, and Hc40 - double SCP-like domain) (Yatsuda et al., 2003). In contrast, in the current study of *H. contortus*, we detected 52 nematode-specific SCP/TAPS proteins. As expected, the majority (71.2%) of them uniquely and/or abundantly represent the parasitic stages (L4: $n = 26$; Af: $n = 29$; Am: $n = 26$) and, thus, may be immunoregulators or molecules with functions governing the parasite-host interplay.

In conclusion, we investigated ES proteins of key developmental stages/sexes (i.e., L3, L4, Af and Am) of *H. contortus* using short-term maintenance culture combined with a high throughput LC-MS/MS-based proteomic approach. We confidently identified a total of 878 ES proteins without intracellular protein contamination. Bioinformatic analyses revealed substantial variation in protein composition and abundance between free-living and parasitic stages, particularly for molecules which are likely involved in nutrient digestion, acquisition and parasite-host interactions. The results provide new insights into the molecular biology of a highly significant parasitic nematode, *H. contortus*, and might support the discovery of novel drug and vaccine targets. The data sets produced provide a useful resource for scientists working on multi-omics of parasitic worms of animals.

3.5. References

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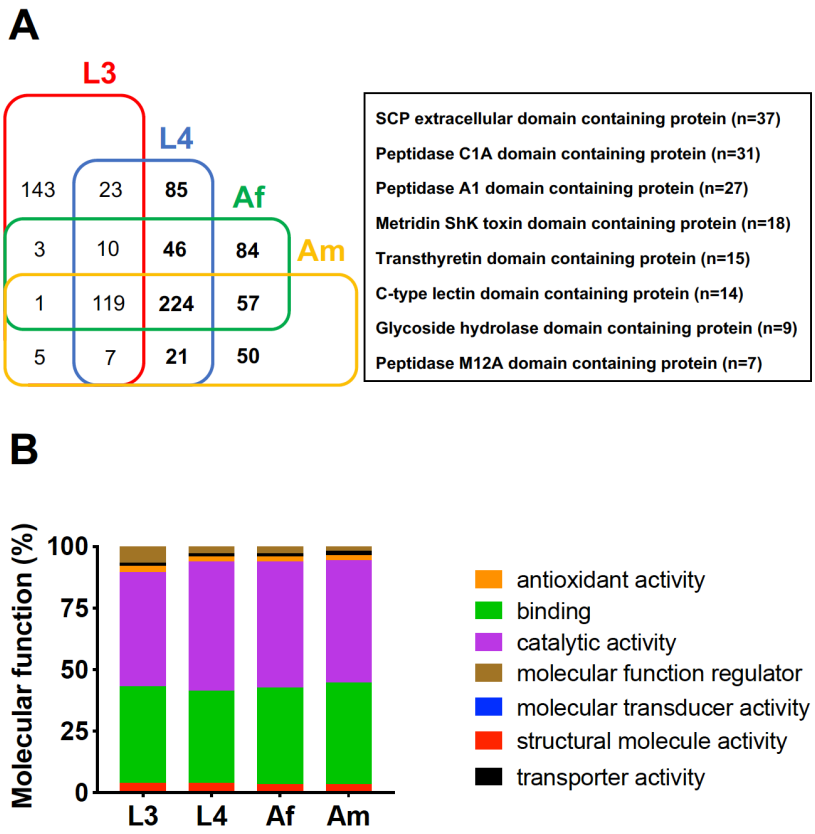


Fig. 3.1. (A) Venn diagrams showing the numbers of quantified proteins unique to or shared by different developmental stages of *Haemonchus contortus* [i.e., third-stage (L3), fourth-stage (L4) larvae, female (Af) and male (Am) adults]. (B) The distribution of the molecular functions (Gene Ontology (GO) level 2) of proteins quantified in each stage. Distribution is presented as a percentage of the total number of proteins identified in a particular stage or sex, to allow quantitative comparisons among different developmental stages/sexes of *H. contortus*.

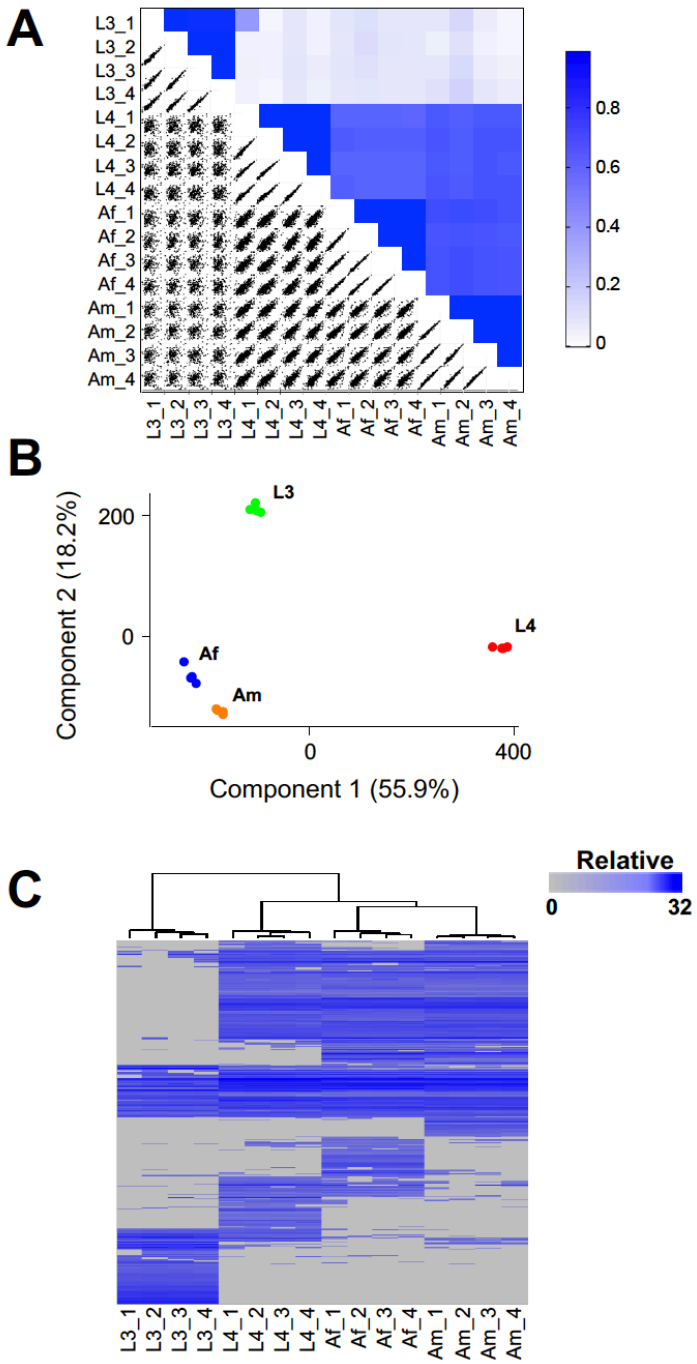


Fig. 3.2. (A) Multi-scatter plots showing the correlation between biological replicates of proteomic differences in developmental stages/sexes of *Haemonchus contortus* upon pairwise comparison. Dark blue represents a high Pearson's correlation between samples. (B) Principal component analysis (PCA) of the secretome of *H. contortus* representing third-stage (L3), fourth-stage (L4) larvae and female (Af) and male (Am) adults. (C) Hierarchical clustering (columns) revealed three distinct clusters comprising the four replicates from their original sample groups. Normalised protein abundance is shown in a grey to blue scale - depicting low to high protein abundance.

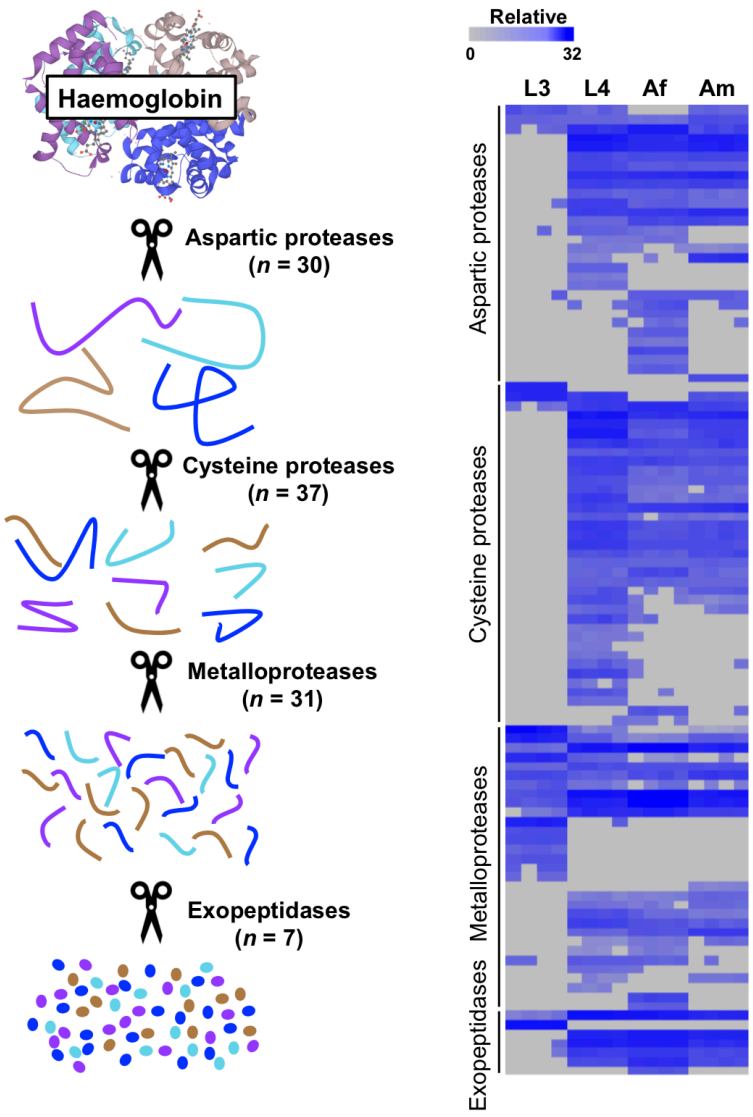


Fig. 3.3. The haemoglobin degradation-related peptidases predicted from the secretome of *Haemonchus contortus* [i.e., third-stage (L3), fourth-stage (L4) larvae, female (Af) and male (Am) adults]. The numbers of peptidases were displayed in the proposed proteolytic cascade responsible for haemoglobin degregation in the gut of haematophagous nematodes (based on Williamson et al., 2003). Normalised protein abundance (columns) is shown in a grey to blue scale - depicting low to high protein abundance.

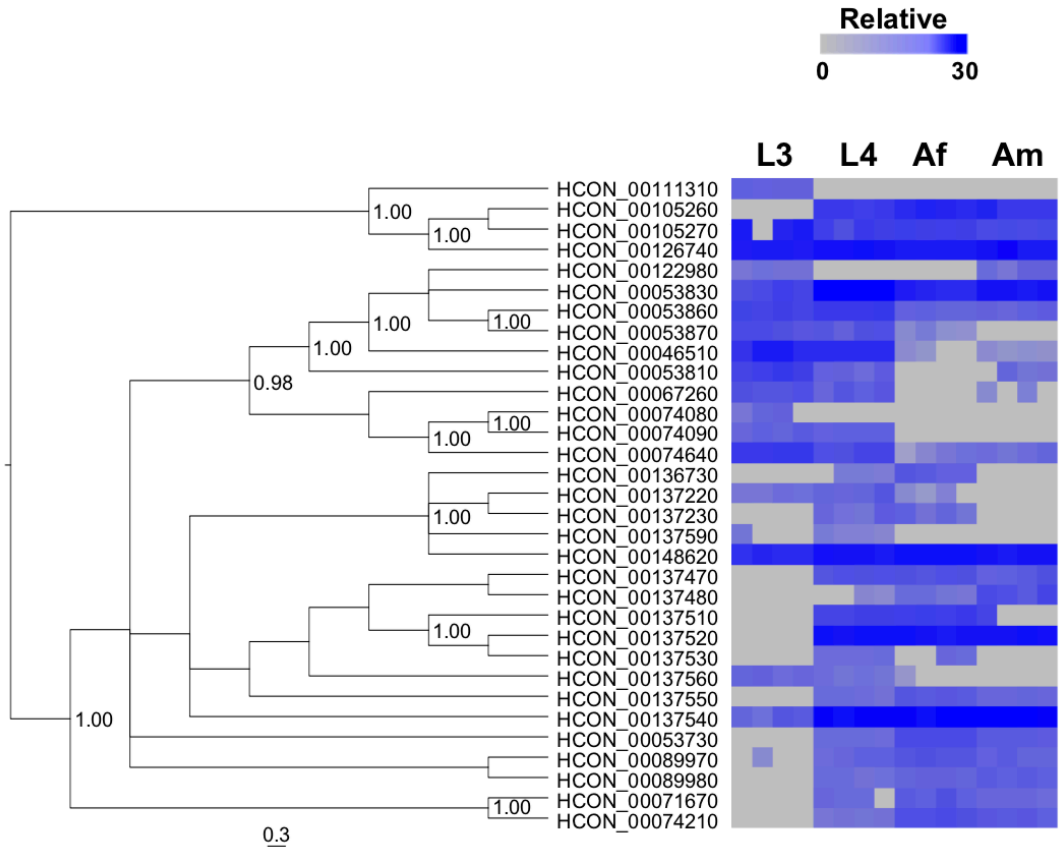


Fig. 3.4. Phylogenetic relationships of the identified transthyretin-like proteins from the secretome of *Haemonchus contortus* [i.e., third-stage (L3), fourth-stage (L4) larvae, female (Af) and male (Am) adults]. Nodal support is given as a posterior probability value. Normalised average protein abundance (columns) is shown in a grey to blue scale - depicting low to high protein abundance.

Supplementary Table S3.1. Complete list of excretory/secretory proteins identified in different developmental stages/sexes of *Haemonchus contortus* (i.e., eggs; third-stage and fourth-stage larvae; female and male adults).

Supplementary Table S3.2. The number of excretory/secretory proteins quantified and inferred to be involved in a particular molecular function (levels 2 and 3) in individual stages/sexes of *Haemonchus contortus* (i.e., third-stage and fourth-stage larvae; and female and male adults), according to the Gene Ontology (GO) categories.

Supplementary Table S3.3. The numbers of ES proteins quantified and inferred to be involved in particular biological processes (levels 2 and 3) in individual stages/sexes of *Haemonchus contortus* (i.e. third- and fourth-stage larvae; and female and male adults), according to Gene Ontology (GO) categories.

Supplementary Table S3.4. The numbers of ES proteins quantified and inferred to be involved in a particular cell component (levels 2 and 3) in individual stages/sexes of *Haemonchus contortus* (i.e. third- and fourth-stage larvae; and female and male adults), according to Gene Ontology (GO) categories.

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Chapter 4. Developmental lipidome of *Haemonchus contortus*

Abstract

Lipids play crucial roles in the biology of organisms, particularly relating to cellular membranes, energy storage, and intra- or inter-cellular signalling. Despite recent expansion of the lipidomics field, very little is known about the biology of lipids in metzoan pathogens, and, to date, there has been no global lipidomic study of a parasitic nematode. Using *Haemonchus contortus* (barber's pole worm) as a model, we describe the first known global lipidome for a parasitic nematode via high throughput LC-MS/MS-based lipidomics. We identified a total of 554 lipid species across four lipid categories, and 18 lipid classes exhibited alterations among six developmental stages (eggs; L3 and exsheathed L3 (xL3) and L4 larval stages; female and male adults) of *H. contortus*. The lipid composition and abundance of *H. contortus* changed significantly during the transition from free-living (egg, L3 and xL3) to parasitic (L4 and adult) stages. The three main changes observed were: (i) decreased synthesis of triradylglycerols; (ii) increased glycerophospholipids (predominantly phosphatidylethanolamine and glycerophosphocholine); and (iii) a 'cooperative' modulation of ether-linked lipids and saturated fatty acids. These changes suggest specific adaptations, in terms of nutrient acquisition, metabolism and development, as the nematode makes its transition to the parasitic stage inside the host animal. This lipidomic data set serves as a stimulus for studies to understand lipid biology in parasitic worms, and their roles in parasite-host interactions and disease processes.

4.1. Introduction

Lipids are a group of hydrophobic or amphipathic small molecules that play essential structural and functional roles in biology, particularly in relation to cellular membranes, energy storage, and intra- and intercellular signalling (Vance et al., 2008). According to their chemical structures and biosynthetic origins, lipids can be grouped into eight major categories, i.e., fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SR) and polyketides (PK) (Fahy et al., 2005). Together with nucleic acids, proteins and other molecules, lipids can regulate complex biochemical networks of cells and tissues to effect cellular homeostasis and differentiation (Kniazeva et al., 2004; Vance et al., 2008).

An increasing emphasis is being placed on the characterisation of the lipid compositions of tissues and cells, and alterations in lipid profiles in organisms of interest under different physiological or pathological conditions (Han, 2016; Yang and Han, 2016; Rustam and Reid, 2018). Accordingly, lipidomics has become an important sub-discipline of systems biology (Subramaniam et al., 2011), devoted to the comprehensive study of global cellular lipid profiles and their key roles in biological networks and metabolic pathways (Kishimoto et al., 2001; Han and Gross, 2003). Despite the expansion of the lipidomics field and the importance of lipids in relation to understanding disease processes (Tam et al., 2013), and the molecular and biochemical interactions between pathogens and their hosts (Wenk, 2006), very little is known about the biology of lipids in parasitic worms.

Most studies of parastic worms have used thin-layer and gas chromatography to identify lipids (Longworth et al., 1987; Smith et al., 1996; Ghosh et al., 2010), but these techniques have limited capacity to resolve lipid composition. Recently, using a direct infusion quadrupole time-of-flight (Q-TOF) mass spectrometry technique, phospholipid profiles of the filarioid nematodes *Onchocerca volvulus*, *Onchocerca ochengi* and *Litomosoides sigmodontis* were characterised (Wewer et al., 2017). However, to date, there has been no global lipidomic study of a parasitic nematode.

Parasitic worms need to continuously adapt to a changing environment within and outside of a host and need to deal with the immune attack from the host (Maizels and McSorley, 2016). Lipid species such as steroids, FA and GP are the crucial building

blocks of biological membranes (Watts and Ristow, 2017). Therefore, it is likely that lipids, together with other molecules including proteins and glycoproteins, play key roles in parasite invasion and persistence in host tissues as well as cross-talk between parasite and host animal, and might constitute potential targets for new interventions against parasitic worms.

In this study, we defined the first known global lipidome for a parasitic nematode. We explored *Haemonchus contortus* (barber's pole worm), one of the most pathogenic nematodes of ruminant animals (Gasser and von Samson-Himmelstjerna, 2016), and characterised the alterations in developmental lipidomic profiles for this worm using LC-MS/MS.

4.2. Materials and methods

4.2.1. Haemonchus contortus and its procurement

Six developmental stages (eggs; L3s, exsheathed L3s (xL3s) and L4s; female (Af) and male (Am) adults) of *H. contortus* were produced as described previously (Campbell et al., 2008; Schwarz et al., 2013) with animal ethics approval (no. 1613878) from the University of Melbourne, Victoria, Australia. Eggs were collected from experimentally infected sheep. L3s were cultured (27 °C), for 7 days, from faeces containing eggs; xL3s were produced in vitro (Preston et al., 2015); L4s and adults (Af and Am) were collected from abomasa 9 and 28 days, respectively, following inoculation of sheep. For each stage/sex, four biological replicates of each life stage and two technical replicates were collected, washed extensively (five times) in large volumes of physiological saline (pH 7.0), pelleted and then frozen at – 80 °C until lipidomic analysis.

4.2.2. Tissue homogenization and lipid extraction

Individual life stages/sexes of *H. contortus* were lyophilised in a benchtop manifold freeze-drier before extraction. Freeze-dried samples (3 mg per stage) were individually transferred to an Eppendorf tube (1.5 ml) containing 200 µL of ice-cold 40% v/v methanol. Samples were homogenized with 100 µL of 0.5 mm zirconium oxide beads

(ZROB05, Next Advance, USA) in a Bead Bullet Blender (Next Advance) twice for 3 min (speed set at 10). Blank tubes with water were processed as controls. Lipids were extracted using an established method (Folch et al., 1957). In brief, 400 µL of chloroform/methanol (2/1, v/v) were added to each tube. The tubes were vortexed for 60 s and centrifuged at 3,000 *g* and 22-24 °C for 10 min. The lower organic phase was then transferred to a new Eppendorf tube, and 400 µL of 100% chloroform was added, vortexed (60 s) and centrifuged as before. The lower organic phase was collected and pooled with the organic phase from the first extraction. The pooled lipid samples were then dried in a SpeedyVac (4000 *g* for 45 min), and lipid pellets re-suspended in 10 µL of isopropanol/methanol/chloroform (4/2/1, v/v/v) containing 0.01% butylated hydroxytoluene (BHT), then diluted with 990 µL of 100% methanol prior to analysis.

4.2.3. LC-MS/MS analysis

All samples were analysed by reverse-phase (RP)-ultra high-performance liquid chromatography (UHPLC)-electrospray ionisation (ESI)-MS and -MS/MS using an Orbitrap Fusion Lumos mass spectrometer coupled to an Ultimate 3000 UHPLC (Thermo Fisher Scientific, San Jose, CA, USA). Solvent A was 6/4 (v/v) acetonitrile/water and solvent B was 9/1 (v/v) isopropanol/acetonitrile; both solvents A and B contained 10 mM ammonium acetate. Each sample (injection volume: 6 µL) was injected into a HSS T3 C18 column (1 mm I.D., x 100 mm, 1.8 µm, 100 Å; Waters, Ireland) at 40 °C at a flow rate of 30 µL/min for 1 min using 30% solvent B. During separation, the percentage of solvent B was increased from 30% to 60% in 1 min, from 60% to 78% in 12 min, from 78% to 91% in 10 min, and from 91% to 97% in 31 min. Subsequently, the percentage of solvent B was increased to 99.5% in 0.1 min then maintained at 99.5% for 4.9 min. Finally, the percentage of solvent B was decreased to 30% in 1 min. A wash-run followed for column cleaning and re-equilibration.

All MS experiments were performed using a Heated Electrospray Ionisation (HESI) source. The spray voltages were 3.5 kV in positive ionisation mode and 2.5 kV in negative ionisation mode. In both polarities, the flow rates of sheath and auxiliary gases were 10 and 5 (arbitrary units), respectively; the capillary and heater temperatures were maintained at 275 °C and 85 °C, respectively, and the S-Lens radio frequency (RF) level

was set at 50%. From 0 to 27 min, positive and negative ionisation mode top speed data-dependent scans were alternated, with each having a cycle time of 1 s. In the positive ionisation mode, full scan MS spectra were acquired in the Orbitrap at a mass resolving power of 120,000 (at m/z 200) across an m/z range of 200–1500 using quadrupole isolation, an automatic gain control (AGC) target of 4e5 and a maximum injection time of 50 milliseconds. Top speed data-dependent, high energy collisional dissociation (HCD)-MS/MS was performed at a mass resolving power of 15,000 (at m/z 200), a normalized collision energy (NCE) of 27%, an m/z isolation window of 1, a maximum injection time of 35 milliseconds and an AGC target of 5e4. For improved structural characterisation of glycerophosphocholine (PC) lipid ions, a data-dependant product ion (m/z 184.0733) triggered collision-induced dissociation (CID) MS/MS scan was performed using a *q* value of 0.25 and a NCE of 30%, with other settings the same as that for HCD MS/MS. In the negative ionisation mode, full scan MS and HCD-MS/MS were performed with the same settings as in positive mode, except that quadrupole isolation was disabled in the full MS scan and that the NCE was set at 30%. From 27 to 61 min, only positive ionisation mode scans were acquired, and the cycle time was set at 2 s. MS and HCD-MS/MS conditions were the same as above. For improved structural characterisation of triradylglycerol (TG) lipids, the FA + NH_3 neutral loss (Supplementary Table S4.1) product ions observed by HCD-MS/MS were used to trigger the acquisition of the top three data-dependent CID MS^3 scans, using a mass resolving power of 15,000 (at m/z 200), a NCE of 30%, an m/z isolation window of 1, a maximum injection time of 35 milliseconds, an AGC target of 5e4 and a *q* value of 0.25.

4.2.4. Identification and quantification of lipids and statistical analysis

MS data were processed using LipidSearch software v.4.1.30 SP1 (Thermo Scientific) (Narvaez-Rivas and Zhang, 2016). Key processing parameters were: target database, general; precursor tolerance, 5 ppm; product tolerance, 5 ppm; product ion threshold, 1%; m-score threshold, 2; quantification m/z tolerance, ±5 ppm; quantification retention time range, ±1 min; use of main isomer filter and identification quality filters A, B and C; adduct ions, +H and $+NH_4$ for positive ionisation mode, and −H and $+CH_3COO$ for negative ionisation mode. All lipid classes in the database within LipidSearch were

selected for the search. The same lipid annotations within ±0.4 min were merged into the aligned results. Unassigned peak areas were calculated for relative quantification and alignment. The shorthand notation used for lipid classification and structural representation follows the nomenclature proposed previously (Liebisch et al., 2013; Ryan and Reid, 2016). To filter false identifications, aligned lipids were examined manually. For TG lipids, a grade A identification was required in at least one life stage, while a grade A, B or C was required for monoradylglycerol (MG) lipids in at least one life stage. For other lipid classes, a grade A or B identification was required in at least one life stage. For all lipids, a signal to noise ratio (S/Z) > 10 and a peak area > 1e7 were required in at least one life stage. All lipid LC-MS, features were manually inspected and re-integrated when needed. To compare the relative abundance of lipids among different stages of *H. contortus*, the ion abundances of corresponding lipid categories and classes were expressed as a percentage of the total ion abundance for the total lipidome, whereas the ion abundances of corresponding lipid species were expressed as a percentage of the total ion abundance for a given lipid class. Venn diagrams of the lipidome of *H. contortus* were produced using the R package v.3.3.1. One-way ANOVA was performed for multiple group comparisons by using GraphPad Prism 6.0 software (GraphPad, La Jolla, United States). Statistical significance was set at $P < 0.05$.

4.3. Results

4.3.1. Haemonchus contortus lipidome

Based on the MS/MS fragmentation patterns, a total of 554 unique lipid species representing four lipid categories (i.e., GL, GP, SP and ST) and 18 lipid classes were identified in *H. contortus* using LipidSearch (Table 4.1). For all samples analysed, > 90% of lipid species belonging to the categories GL and GP represented 14 lipid classes. By contrast, < 10% of species belonging to the categories SP and ST represented the four other classes. Most species (n = 236) were in class TG, followed by some in classes phosphatidylethanolamines (PE; n = 84) and glycerophosphocholines (PC; n = 65). Few species were in classes lysophosphatidylglycerols (LPG; n = 3) and phosphatidylglycerols (PG; n = 3) (Table 4.1).

Among all developmental stages, the greatest number of lipid species were identified in the egg stage (n = 382), followed by Af (n = 375) and Am (n = 356), whereas only 327 lipids were found in L3s. The identities of lipid species that were shared among different developmental stages (i.e., eggs, L3s+xL3s, L4s and adults) are given in Fig. 4.1. Most of the lipid species identified (n = 437, 80.3%) were shared by at least two developmental stages of *H. contortus*, of which 225 identities (40.6%) were shared by all stages studied. These shared lipid species are mainly of the classes TG (n = 97) and PE (n = 37), within which FA with 18 carbons (i.e., 18:0, 18:1, 18:2 and 18:3) were most common (53.4%). In addition, L3s and xL3s contained most stage-specific lipid species (n = 65, 11.7%), with TG being the major species (n = 39). The full lists of lipid species identified for each life stage are given in Supplementary Table S4.2.

4.3.2. Alterations in relative lipid abundance during H. contortus development

Relative quantification showed that GL (13.9-82.9%) and GP (16.1-82.0%) lipids were the two most abundant categories, whereas SP and ST lipids represented < 4% of the total ion abundance in each stage (Fig. 4.2). Considering the abundant lipid classes, TG was abundant in most developmental stages (37.5-80.9%), except for the Am stage (11.0%), with a down-regulation in free-living (egg and L3) compared with parasitic (L4, Af and Am) stages. TG showed the most significant decrease from L3s (80.9%) to L4s (37.5%, P <0.05) and Am (11.0%, P < 0.01) (Fig. 4.3). An analysis of individual lipid species showed that those containing odd-numbered carbon chain length FA (e.g., 13:0, 15:0 and 17:0) contributed significantly to a decrease in TG (Fig. 4.3). Interestingly, individual lipid species such as TG (15:0_15:0_18:1) and TG (15:0_15:0_15:0) were detected predominantly only in L3s and xL3s. PC (9.3-54.9%) and PE (6.0-22.9%) were the two most abundant lipid classes of GP, exhibiting higher levels in parasitic than free-living stages (Fig. 4.4 and 4.5). The largest change in PC occurred from L3s (9.3%) to Am (54.9%) (P < 0.01). Similar to PC, PE increased significantly from L3s (6.0%) to Am (22.9%) (P < 0.01). Individual lipid species, which contain long-chain FA (> 36 total carbons) contributed significantly to an increase in PE and PC during the development from egg to adult *H. contortus* (Fig. 4.4 and 4.5). Noteworthily, individual lipids PE (P-18:0_20:3) and PE (P-18:0_20:4) exhibited an opposite pattern with relatively higher

levels in L3s and xL3s and lower levels in the adult stages (Fig. 4.5). Taken together, the relative quantification data showed marked changes in relative lipid abundance throughout *H. contortus* development (Supplementary Fig. S4.1-3). The full lists of quantified lipid species for each life stage are given in Supplementary Table S4.2.

4.3.3. FA composition in different developmental stages of H. contortus

Haemonchus contortus lipids contain a high level of unsaturated FA and long-chain FA (> 12 carbons), representing 56.3% and 98.1% of the total FA composition, respectively (Table 4.2). An analysis of saturated FA and ether-linked lipids showed that saturated FA mainly represented TG (n = 24), followed by PE (n = 17) (Supplementary Table S4.3), whereas ether-linked lipids were predominantly PE (n = 43), TG (n = 15) and PC (n = 13), followed by LPE (n = 4), PI (n = 3) and LPC (n = 2) (Supplementary Table S4.4). There was an inverse relationship between saturated FA lipids and ether-linked lipids in particular stages of *H. contortus*. L3s and xL3s with high levels (11.5-18.0%) of saturated FA had low levels (4.5-9.8%) of ether-linked lipids, whereas L4s, Af and Am (parasitic stages) with low levels of saturated FA (1.9-4.2%) had high levels of ether-linked lipids (12.6-20.7%) (Fig. 4.6). The total ion abundance for saturated FA lipids decreased significantly from 18.0% in L3s ($P < 0.01$) and 11.5% in xL3s ($P < 0.05$) to 1.9% in L4s, whereas that for ether-linked lipids increased significantly from 4.5% in L3s to 9.8% in L4s ($P < 0.05$) and 20.7% Am ($P < 0.01$).

4.4. Discussion

Here we used LC-MS/MS to undertake a relative quantification of lipids in *H. contortus* - one of the most important parasitic nematodes of livestock – which now allows comparisons to be drawn with the lipidome of *Caenorhabditis elegans* - the best-studied metazoan organism (free-living nematode) in the same evolutionary clade as *H. contortus* (see Cantacessi et al., 2012). We reveal that, in spite of similarities in the nature and extent of the lipidome between these nematodes, there are some significant differences in lipid classes and species that likely relate to unique biology and adaptations to the environments in which they live, develop and reproduce. Similarities in the

lipidome of *H. contortus* and *Caenorhabditis elegans* relate mainly to key structural and functional/metabolic/biochemical components likely to be common among nematodes, irrespective of whether they are parasitic or free-living.

Consistent with *Caenorhabditis elegans*, GP lipids (mainly PC and PE) of *H. contortus* are abundant in all stages/sexes, exhibiting an increase in abundance during development (Fig. 4.2). These lipids, as well as SP lipids, are building blocks of membranes (Witting and Schmitt-Kopplin, 2016; Watts and Ristow, 2017). As membranes are consumed in multiple cellular processes (e.g., intracellular trafficking, β-oxidation of FA and cellular signalling), there is a constant turnover (i.e., synthesis and degradation) and remodelling of GP (Hou et al., 2014; Dancy et al., 2015). A previous study of *Caenorhabditis elegans* revealed that the replacement of the majority of membrane lipids occurs daily, with ~2% of GP species being replaced every hour (Dancy et al., 2015). If the same condition applies, one might expect dramatic remodelling and developmental regulation of membrane lipids in *H. contortus* and other parasitic nematodes, particularly during invasion of and adaption in the host. Notably, in *H. contortus,* PC lipids are most abundant in Am worms, whereas TG predominate in all other stages. Further work should explore the distribution of these lipids in membranes in different cell and tissue types of *H. contortus*.

Phosphatidylethanolamines (PE) represent the largest group of ether-linked lipid species in *Caenorhabditis elegans* (see Satouchi et al., 1993; Drechsler et al., 2016; Shi et al., 2016). In *H. contortus*, ether-linked lipids are represented mainly in PE (n = 43), followed by TG (n = 15) and PC (n = 13) (Supplementary Table S4.4). *Caenorhabditis elegans* tends to accumulate cellular saturated FA levels, in particular 18:0 FA, likely to compensate for the absence of ether lipids and improve membrane rigidity (Shi et al., 2016). The latter authors suggested that this compensation is partially mediated by the increasing in de novo-FA synthesis and transcriptional reduction of lipid desaturase activity. Although similar compensatory alterations of saturated FA exist in *H. contortus* (Fig. 4.6), the present study did not show an accumulation of 18:0 FA. Whether the regulation of ether-linked lipids and saturated FA during the development of *H. contortus* relates to membrane rigidity remains to be elucidated.

Ether-linked compounds of phospholipids are also of considerable interest due to their unique structure (i.e., a fatty acyl group, typically a saturated FA) that is linked to the sn1

position of glycerol backbone, which supports key cellular functions such as membrane fluidity, anti-oxidation and lipid signalling (Wallner and Schmitz, 2011; Braverman and Moser, 2012). Previous phenotypic analysis of the ether lipid biosynthetic mutant *Caenorhabditis elegans* strains showed a low tolerance to oxidative stress, short lifespan, sensitivity to low temperatures (10 and 15 °C) and reduced fecundity (Shi et al., 2016). Importantly, ether-linked lipids contribute significantly to the physical rigidity of the worm surface (cuticle) and protection of the worm itself, which is in contact with host cells and tissues.

The features of the *H. contortus* lipidome that are distinct from *Caenorhabditis elegans* likely reflect the parasite's distinct biology, and suggest that particular lipid classes or species are essential for parasitism. Indeed, in *H. contortus*, there is a relationship between the abundance of particular lipid species/classes in and developmental stage of the worm, which indicates specific adaptations of nutrient acquisition, metabolism and development to the environment outside and inside of the host animal, as it transitions through its life cycle.

In *H. contortus*, energy storage lipids (TG) represent the most abundant lipid class and undergo the greatest change (Fig. 4.2A). Unlike the TG plateau (40-55%) seen in all developmental stages of *Caenorhabditis elegans* (see Cooper and van Gundy, 1971; Ashrafi, 2007), there are substantial changes in the TG profile during *H. contortus* development. In the free-living stages of *H. contortus* (i.e., egg, L3 and xL3), TG represent more than half of all identified lipids, reaching a maximum of 80.9% in the L3 stage, and then decreasing significantly to 37.5% in the L4 stage and 11.0% in the Am. This "down-regulation" of this major lipid class as the nematode develops to the adult stage seems to relate to a reduction in lipid metabolism, a statement supported by independent transcriptomic data (Laing et al., 2013).

Although the lipid metabolism of parasitic nematodes is poorly understood, it is thought that these worms are not able to synthesise long-chain FA and sterols de novo (Harder, 2016). To meet the substantial energy requirement of continuous growth, parasitic nematodes would appear to extract most of their nutrients (including amino acids, nucleic acids and lipids) from the host's tissues or diet (parasitic phase) or from the environment (free-living phase) (Harder, 2016). In the case of *H. contortus*, nutrient uptake would be predominantly via blood in the adult stage. The proposed consumption

of lipids by parasitic nematodes might be explained by the negative relationship between some host lipids and the numbers of worms infecting a host. For instance, studies have shown significantly lower serum lipid levels (total TG and cholesterol) in calves infected with low or high numbers of nematodes (i.e., species of *Haemonchus*, *Cooperia*, *Trichostrongylus* and *Oesophagostomum*) (see de Cezaro et al., 2016). Similar observations have been made in guinea pigs infected with *Ascaris suum* (Biadun, 1990) and in human patients with *Strongyloides stercoralis* infection (de Jesus Ines et al., 2017). Based on these findings, it appears that parasitic nematodes directly consume lipids from within the host animal.

It is likely that *H. contortus* regulates its lipidome to adapt to the host environment. Once established in the stomach (abomasum), host TG lipids likely form an essential energy source for the parasite. TG abundance in Af (39.0%) is higher than in Am (11.0%). Considering the reproductive potential of *H. contortus* Af worms (up to 4000 eggs per day) (Fleming, 1988), there is a massive need for energy to produce and nurture the eggs in utero. The abundance of TG in eggs (53.4%) in the reproductive tract is likely the major source of energy for egg production in Af. In *Caenorhabditis elegans*, accumulated fat in embryos is processed in a rapid and effective manner (McGhee, 2007). During the peak of egg laying, hermaphrodites ingest large numbers of bacteria and are efficient at converting the accumulated body mass into embryos each day (McGhee, 2007). It is likely that Af of *H. contortus* actively store "lipid energy" in eggs to prepare for the free-living stage of the life cycle. Interestingly, esterification appears to differ among nematodes. In *H. contortus*, volatile FA have limited esterification, which contrasts *Ascaris*, for example, where esterified volatile FA such as 2-methylbutyrate, 2-methylvalerate are abundant in eggs (Barrett et al., 1970). Future work should explore the reasons for these differences and the roles of these FA in different species of nematodes.

Compared with *Caenorhabditis elegans* (~40% in total lipids) (Tanaka et al., 1996; Watts and Browse, 2002), the *H. contortus* lipidome contains an abundance of saturated FA (~44% in total lipids) (Tanaka et al., 1996). According to the homeoviscous theory of membrane adaptation, ectothermic animals including nematodes tend to regulate membrane saturation levels to adapt to temperature changes in the environment (Sinensky, 1974). Previous studies have indicated that *Caenorhabditis elegans* adapts to different environmental temperatures by altering its levels of saturated FA (increased

levels at higher temperatures) (Murray et al., 2007; Ma et al., 2015). However, the converse appears to be the case in *H. contortus*, as the saturated FA levels are high in the transition from the free-living phase (i.e., 18.0% at L3 stage; 10 °C) compared with the parasitic phase (i.e., 1.9% at L4, 4.1% at Af and 4.2% at Am stages) in the host animal (37-39 °C) (Fig. 4.3C). Based on current knowledge of the first parasitic nematode lipidome, this observation it is somewhat challenging to explain. Clearly, much more work is needed to find clues to solve this puzzle.

In this report, we describe the first global known lipidome for a parasitic nematode. Using an LC-MS/MS-based lipidomic approach, we directly identified lipids and observed their alterations in six stages of *H. contortus.* We categorised more than 500 lipid species in 18 classes with confidence (based on MS/MS fragmentation patterns), highlighting the exquisite applicability of this approach. The present investigation showed significant differences in lipid abundance in the transition from free-living to parasitic stages, particularly for lipid species likely linked to essential roles in cellular processes and functions (i.e., membrane remodelling, energy storage and signalling regulation), suggesting that parasitic nematodes actively regulate their lipidome to adapt to a parasitic mode of existence. The present lipidomic data set should complement and enhance the value of genomics, transcriptomics and proteomics via multi-omic data integration, to better understand parasitic worms, host interactions and disease processes, and to stimulate work toward developing new anthelmintic interventions.

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Table 4.1. Summary of the number of lipid species identified in extracts derived from different developmental stages of *Haemonchus contortus*.

Lipid category/class	Eggs	L3s	xL3s	L4s	Adult female (Af)	Adult male (Am)	Total lipids
Glycerolipids							
MG	9	4	4	2	9	5	10
DG	29	21	14	18	28	16	39
TG	163	151	159	155	150	148	236
Glycerophospholipids							
PC	42	37	34	46	51	49	65
PE	62	41	45	51	52	54	84
PG	2	3	3	1	1	1	3
PI	14	12	13	14	16	15	17
PS	1	3	3	3	0	1	4
CL	4	4	5	7	7	6	7
LPC	12	9	9	14	14	14	14
LPE	9	7	6	9	9	11	11
LPG	2	3	0	0	0	0	3
LPI	5	4	2	2	5	6	7
LPS	2	4	4	3	0	2	4
Sphingolipids							
SM	6	10	9	6	9	7	18
Cer	8	5	7	8	10	7	11
HexCer	3	5	7	3	3	4	10
Sterol lipids							
CE	9	4	6	11	11	10	11
Total	382	327	330	353	375	356	554

MG: Monoradylglycerols; DG: Diradylglycerols; TG: Triradylglycerols; PC: Glycerophosphocholines; PE: Glycerophosphoethanolamines; PG: Glycerophosphoglycerols; PI: Glycerophosphoinositols; PS: Glycerophosphoserines; CL: Cardiolipins; For LPC, LPE, LPG, LPI and LPS, prefix 'L' was added for each lysophospholipid class; SM: Sphingomyelins; Cer: Ceramide; HexCer: Hexosylceramide; CE: Cholesteryl ester; xL3: exsheathed L3.

Table 4.2. Fatty acid (FA) composition of lipids species identified in the lipidome of *Haemonchus contortus*.

Lipid category	Saturated (%)		Unsaturated (%)		Odd-chain FA (%)	Even-chain FA (%)	Total no. of FA
	Medium-chain FA[a]	Long-chain FA[b]	Medium-chain FA[a]	Long-chain FA[b]			
Glycerolipids	0.7	25.3	1.2	34.1	14.5	46.8	796
Glycerophospholipids	ND	14.3	nd	17.5	4.5	27.3	413
Sphingolipids	ND	3.2	nd	2.8	2.6	3.4	78
Steroid lipids	ND	0.2	nd	0.7	nd	0.8	11
Total	0.7	43.0	1.2	55.1	21.6	78.4	1298

[a]Medium-chain FA contain 6-12 carbons.
[b]Long-chain FA contain more than 12 carbons
nd: not detected

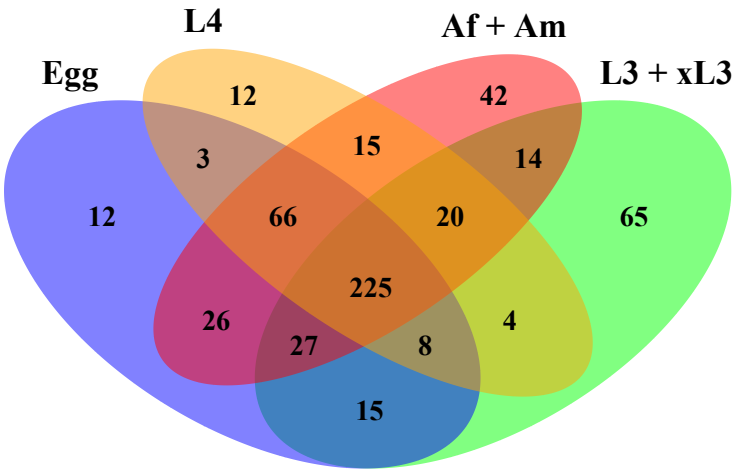


Fig. 4.1. Venn diagram showing the similarity of identified lipid species among different developmental stages of *Haemonchus contortus*, including eggs (Egg), L3s + exsheathed L3s (L3 + xL3), L4, female (Af) and male (Am) adults.

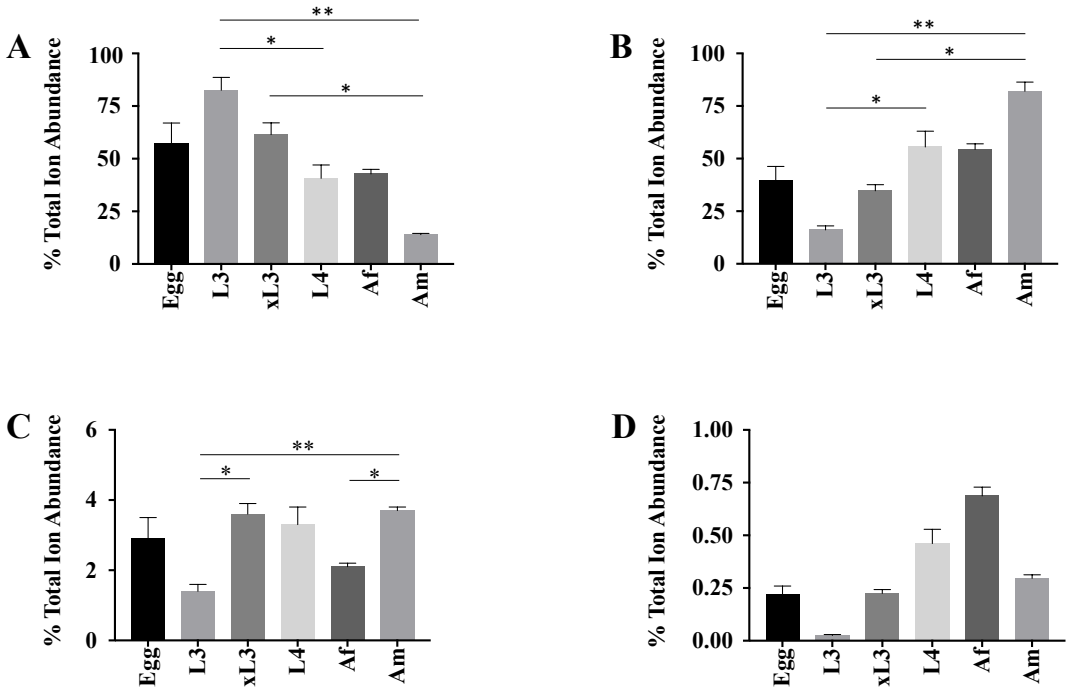


Fig. 4.2. Relative quantification changes of lipid categories from different developmental stages of *Haemonchus contortus*. (A) Glycerolipids; (B) Glycerophospholipids; (C) Sphingolipids; (D) Sterol lipids. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative standard deviation (SD) of the mean (four biological replicates). xL3: exsheathed L3s; Af: female adults; Am: male adults.

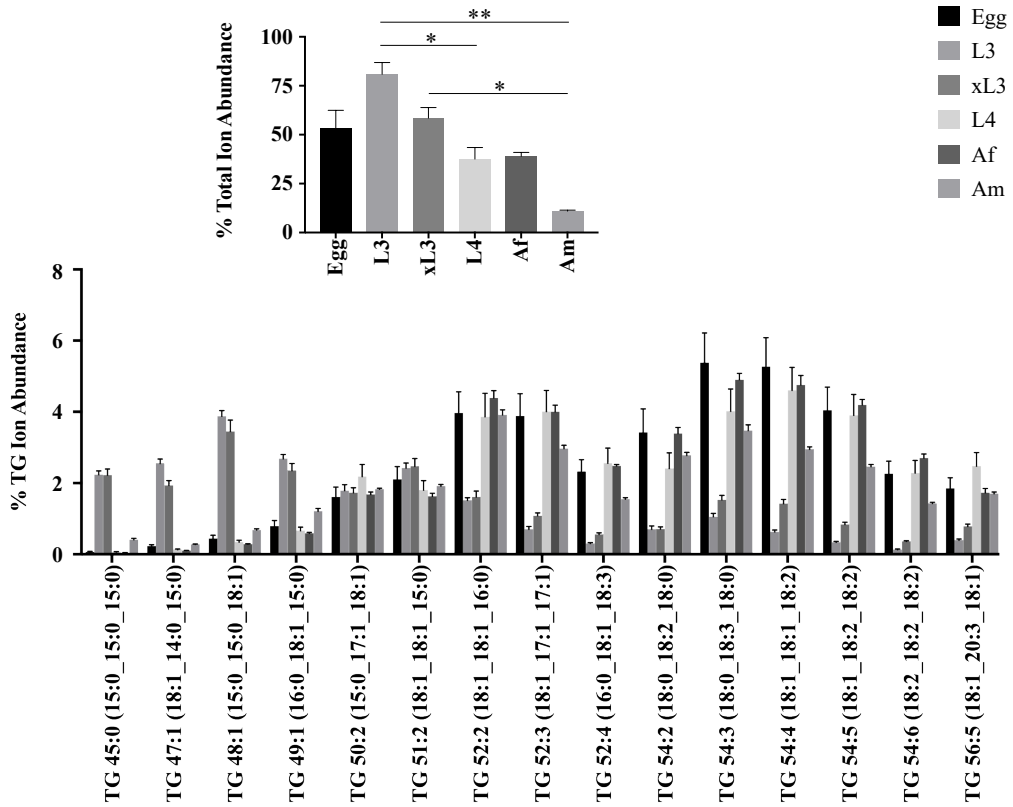


Fig. 4.3. Relative quantification changes of individual triradylglycerol (TG) lipids from different developmental stages of *Haemonchus contortus*. Only lipid species with > 2% ion abundance (at any life stage) are shown. The inset shows the percentage of total lipid ion abundances for TG from distinct stages of *H. contortus*. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative SD of the mean (four biological replicates). xL3: exsheathed L3s; Af: female adults; Am: male adults.

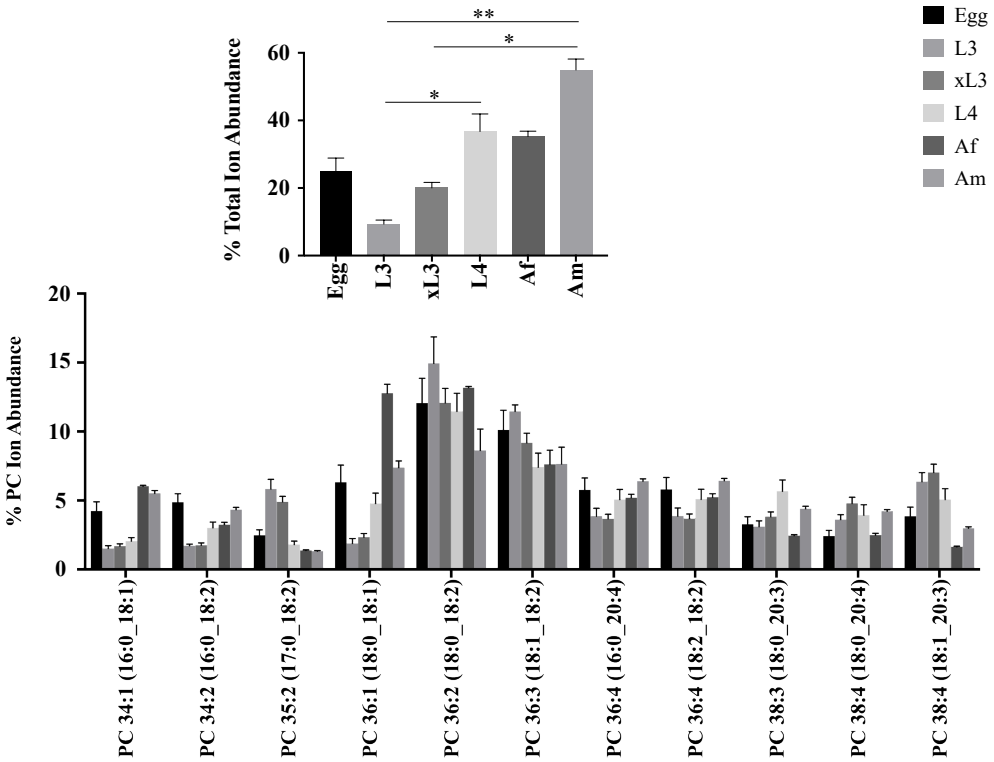


Fig. 4.4. Relative quantification changes of individual glycerophosphocholine (PC) lipids from different developmental stages of *Haemonchus contortus*. Only lipid species with >4% ion abundance (at any stage) are shown. The inset shows the percentage of total lipid ion abundances for PC from distinct stages of *H. contortus*. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative standard deviation (SD) of the mean (four biological replicates). xL3: exsheathed L3s; Af: female adults; Am: male adults.

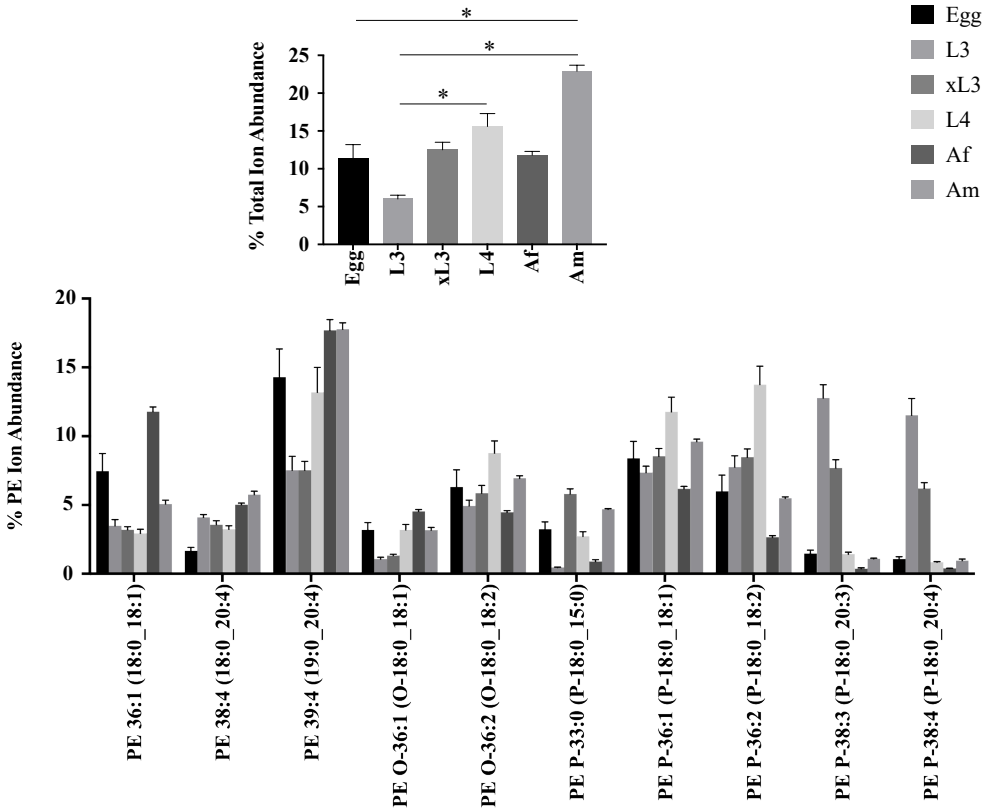


Fig. 4.5. Relative quantification changes of individual phosphatidylethanolamine (PE) lipids from different developmental stages of *Haemonchus contortus*. Only lipid species with > 4% ion abundance (at any life stage) are shown. The inset shows the percentage of total lipid ion abundances of PE from distinct stages of *H. contortus*. Statistical analysis was performed by ANOVA (*$P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative standard deviation (SD) of the mean (four biological replicates). xL3: exsheathed L3s; Af: female adults; Am: male adults.

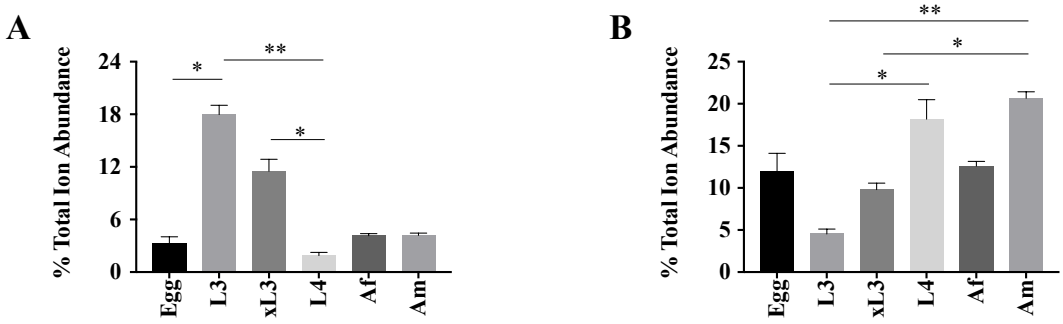
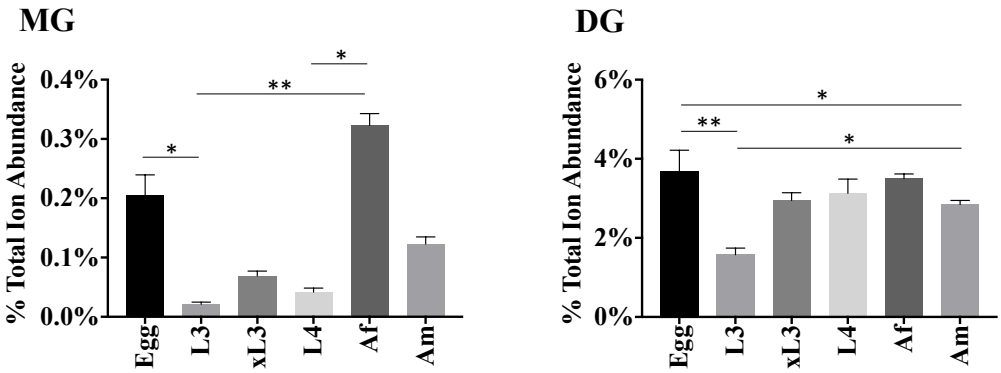
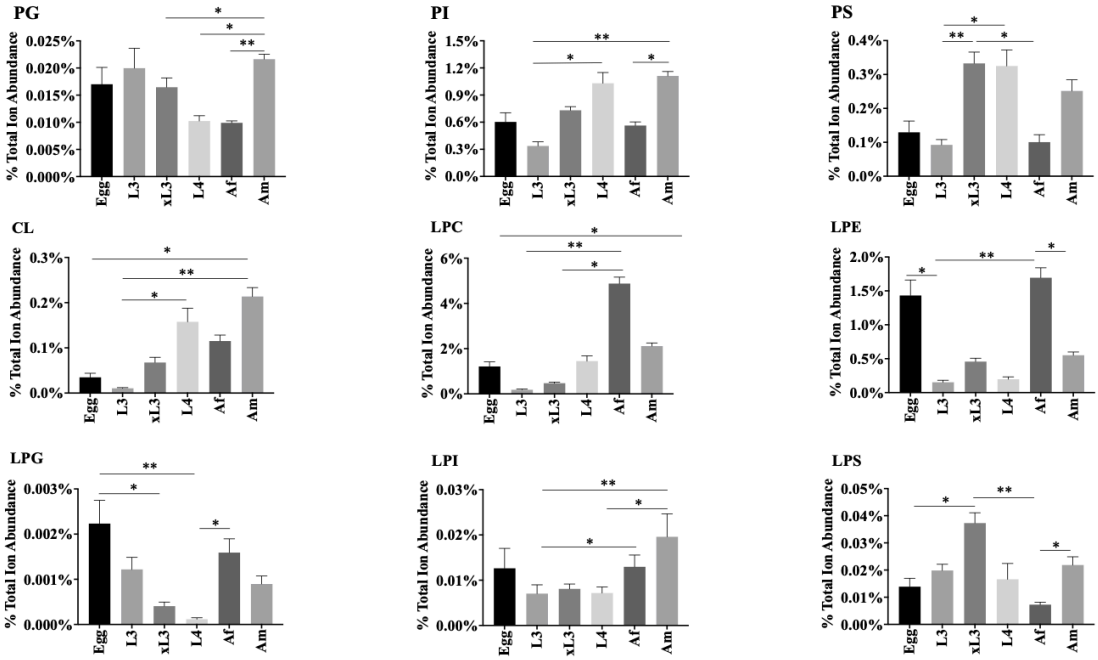


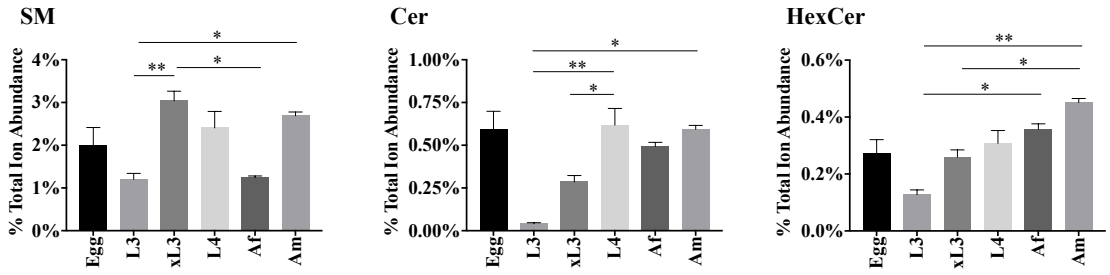
Fig. 4.6. Relative quantification changes of total saturated fatty acids and ether-linked lipid ion abundance from different developmental stages of *Haemonchus contortus*. (A) saturated fatty acids; (B) ether-linked lipids. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± SEM (four biological replicates). xL3: exsheathed L3s; Af: female adults; Am: male adults.



Supplementary Fig. S4.1. Relative quantification changes of monoradylglycerol (MG) and diradylglycerols (DG) from different developmental stages of *Haemonchus contortus*. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative SD (four biological replicates).



Supplementary Fig. S4.2. Relative quantification changes of glycerophosphoglycerols (PG), glycerophosphoinositols (PI), glycerophosphoserines (PS), cardiolipins (CL), lyso-phosphatidylcholine (LPC), -phosphatidylethanolamine (LPE), -glycerophosphoglycerols (LPG), -glycerophosphoinositols (LPI) and -glycerophosphoserines (LPS) from different developmental stages of *Haemonchus contortus*. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative SD (four biological replicates).



Supplementary Fig. S4.3. Relative quantification changes of sphingomyelins (SM), ceramides (Cer) and hexosyl ceramides (HexCer) from different developmental stages of *Haemonchus contortus*. Statistical analysis was performed by ANOVA (* $P < 0.05$; ** $P < 0.01$). Error bars indicate ± relative SD (four biological replicates).

Supplementary Table S4.1. Fatty acid neutral loss in TG lipids.

Supplementary Table S4.2. The full list of lipid species identified in the lipidome of *Haemonchus contortus*.

Supplementary Table S4.3. The list of saturated lipid species identified in the lipidome of *Haemonchus contortus*.

Supplementary Table S4.4. The list of ether-linked lipid species identified in the lipidome of *Haemonchus contortus*.

Chapter 5. Molecular alterations during larval development of *Haemonchus contortus* in vitro are under post-transcriptional control

Abstract

In this study, we explored the molecular alterations in the developmental switch from the L3 to the exsheathed L3 (xL3) and to the L4 stage of *Haemonchus contortus* in vitro using an integrated transcriptomic, proteomic and bioinformatic approach. Totals of 9,754 mRNAs, 88 microRNAs (miRNAs) and 1,591 proteins were identified, and 6,686 miRNA-mRNA pairs inferred in all larval stages studied. Approximately 16% of transcripts in the combined transcriptome (representing all three larval stages) were expressed as proteins, and there were positive correlations (r = 0.39 to 0.44) between mRNA transcription and protein expression in the three distinct developmental stages of the parasite. Of the predicted targets, 1,019 (27.0%) mRNA transcripts were expressed as proteins, and there was a negative correlation (r = -0.60 to -0.50) in the differential mRNA transcription and protein expression between developmental stages upon pairwise comparison. The changes in transcription (mRNA and miRNA) and protein expression from the free-living to the parasitic life cycle phase of *H. contortus* related to enrichments in biological pathways associated with metabolism (e.g., carbohydrate and lipid degradation, and amino acid metabolism), environmental information processing (e.g., signal transduction, signalling molecules and interaction) and/or genetic information processing (e.g., transcription and translation). Specifically, fatty acid degradation, steroid hormone biosynthesis and the Rap1 signalling pathway were suppressed, whereas transcription, translation and protein processing in the endoplasmic reticulum were upregulated during the transition from the free-living L3 to the parasitic xL3 and L4 stages of the nematode in vitro. Dominant post-transcriptional regulation was inferred to elicit these changes, and particular miRNAs (e.g., *hco-miR-34* and *hco-miR-252*) appear to play roles in stress responses and/or environmental adaptations during developmental transitions of *H. contortus*. Taken together, these integrated results provide a comprehensive insight into the developmental biology of this important parasite at the molecular level in vitro. The approach applied here to *H. contortus* can be readily applied to other parasitic nematodes.

5.1. Introduction

The free-living nematode *Caenorhabditis elegans* is one of the best-characterised metazoan organisms (Corsi et al., 2015). Integrated analyses of molecular data sets for this nematode have made it possible to gain deep, global insights into areas such as the developmental and reproductive biology, physiology, biochemistry, genetics and neurobiology of metazoans (Piano et al., 2006; van Assche et al., 2015). By comparison, little is known about these areas for parasitic nematodes. Since *Caenorhabditis elegans* is relatively closely related to the socioeconomically important parasitic nematodes of the order Strongylida (Blaxter et al., 1998), the information and data sets available for *Caenorhabditis elegans* can significantly assist molecular investigations of strongylid nematodes (Gilleard, 2004; Howe et al., 2016a, b). There have been major advances in the genomics and transcriptomics of worms such as *Haemonchus contortus* (barber's pole worm), *Necator americanus* (hookworm) and *Oesophagostomum dentatum* (nodule worm) (see Laing et al., 2013; Schwarz et al., 2013; Tang et al., 2014; Tyagi et al., 2015), allowing novel insights into the systems biology of strongylid nematodes.

Haemonchus contortus is one of the most important parasitic nematodes of livestock animals worldwide (cf. Gasser and von Samson-Himmelstjerna, 2016). This parasite is transmitted orally from contaminated pasture to the host through a relatively complex, 4-week life cycle (Veglia, 1915): eggs are excreted in host faeces; the L1s develop inside the eggs and then hatch (within ~1 day) and moult to the L2 and L3 stages within 1 week; the infective L3s are then ingested by the host, exsheath and, following a histotropic phase, develop through the L4 stage to dioecious adults which both feed on blood from vessels in the stomach wall.

Although some studies have explored changes or differences in mRNAs or proteins in *H. contortus* (see Campbell et al., 2008; Cantacessi et al., 2010; Laing et al., 2013; Schwarz et al., 2013; Wang et al., 2016), no investigation has yet integrated or compared transcriptomic and proteomic profiles during developmental transitions in this nematode or explored regulatory molecular processes. For instance, while small RNAs, such as microRNAs (miRNAs) (Kim, 2005; Grosshans and Filipowicz, 2008), play key roles in regulating genes via mRNA silencing or chromatin modification (Kim, 2005; Grosshans and Filipowicz, 2008), limited information is available for *H. contortus* (see Winter et al.,

2012; Gillan et al., 2017; Gu et al., 2017), particularly in relation to this parasite's developmental biology. Here, we explored mRNA, miRNA and protein alterations in *H. contortus* during the developmental transition from ensheathed L3 to xL3 and then to the L4 stage in vitro, utilising a combination of transcriptomic, proteomic and bioinformatic tools. The goal was to identify key molecular changes associated with this crucial phase of this nematode's development using a data integration approach.

5.2. Materials and methods

5.2.1. Procurement of parasite and storage

Haemonchus contortus (Haecon-5 strain) was maintained in experimental sheep (cf. Schwarz et al., 2013), in accordance with the institutional animal ethics guidelines (permit no. 1613878; The University of Melbourne, Australia). In brief, helminth-free Merino sheep (8 weeks of age) were inoculated with 7,000 L3s of *H. contortus.* Four weeks p.i., faecal samples were collected each day; L3s were produced by incubating faeces at 27 °C for 1 week (cf. Schwarz et al., 2013), purified through two layers of nylon mesh (pore size: 20 µM; Rowe Scientific, Australia) and stored at 10 °C until use.

5.2.2. Exsheathment of L3s and culturing to L4s in vitro

L3s were sterilised and exsheathed by incubation in 0.15% v/v sodium hypochlorite (NaClO) at 37 °C for 20 min (Preston et al., 2015). This method was optimised to consistently achieve an exsheathment rate of ≥ 80% (Preston et al., 2015). xL3s were rapidly washed thrice in sterile physiological saline and twice in sterile Luria Bertani (LB medium) by centrifugation at 600 *g* (5 min) supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 2.5 µg/ml of amphotericin (Fungizone®, antibiotic-antimycotic; cat no. 15240-062; Thermo Fisher Scientific, USA) and 200 µg/ml of gentamicin (Gibco®, cat no. 15710-064; Thermo Fisher Scientific, USA) - supplemented LB was designated LB*. After the last wash, xL3s were suspended in LB* (38 °C) at a density of 6,000 larvae per ml, transferred to six wells in a culture plate (cat no. 4430500N; Orange Scientific, Belgium) and immediately incubated at 38 °C in an

atmosphere of 10% v/v CO_2 in a water-jacketed incubator (model no. 2406 Shel Lab, USA) for 8 h to recover, or 7 days to develop to L4s. Subsequently, all samples were washed thrice in sterile saline (38 °C), snap frozen in liquid nitrogen and stored at – 80 °C.

5.2.3. RNA isolation, RNA-seq and RNA data processing

Total RNA was extracted from five replicates of each L3s, xL3s and L4s (30,000 larvae per replicate) of *H. contortus* using TriPure isolation reagent (cat no. 11667157001; Roche, Germany), and treated with RNase-free TURBO DNase (Ambion®, cat no. AM1907; Thermo Fisher Scientific, USA). Libraries for sequencing were constructed from mRNA and small RNA from L3s, xL3s and L4s using TruSeq Stranded Total RNA Library Prep Kit (Illumina) and TruSeq Small RNA Library Prep Kit (Illumina), respectively. In brief, mRNA was isolated using magnetic beads with Oligo (dT), cleaved into short fragments, transcribed into complementary DNA (cDNA) by reverse synthesis, connected with adaptors and amplified by PCR for mRNA-enriched libraries. For small RNA libraries, RNAs of 20-30 nucleotides (nt) in length were selected by electrophoresis, ligated to adaptors and amplified. All libraries were evaluated using the Agilent 2100 Bioanalyzer and ABI StepOnePlus real-time PCR system, and then sequenced using the Illumina HiSeq™ 4000 platform.

Strand-specific, raw mRNA reads (150 nt in length) were processed to remove adaptors and low-quality reads using Trimmomatic (Bolger et al., 2014). High quality mRNA reads were used for de novo assembly and genomic-guided assembly of transcripts employing the programs Trinity (Grabherr et al., 2011), TopHat2 (Kim et al., 2013) and Cufflinks2 (Trapnell et al., 2010). Genome-guided assembly was assisted using the publicly available genome assembly of *H. contortus* (MHco3 ISE strain) (see Doyle et al., 2018). The Illumina reads were mapped to predicted genes using the program RSEM (Li and Dewey, 2011) to calculate expected read counts. The threshold for mRNA transcription to be recorded was > 1 read counts per million (CPM) in at least five samples.

Strand-specific, high quality short reads (50 nt in length) produced from small RNA sequencing were processed and analysed using a miARma-Seq package (Andrés-León et al., 2016). In brief, the short reads were processed to remove adaptors, reads of low

quality and reads of < 18 nt in length; clean reads were mapped to the genome assembly of *H. contortus* (perfect match) to identify known miRNAs and to predict novel miRNAs. Precursor and mature miRNA sequences representing *H. contortus* as well as *Ascaris suum*, *Brugia malayi*, *Caenorhabditis brenneri*, *Caenorhabditis briggsae*, *Caenorhabditis elegans*, *Caenorhabditis remanei*, *Pristionchus pacificus*, *Panagrellus redivivus* and *Strongyloides ratti* were downloaded from miRBase (release 21; Kozomara and Griffiths-Jones, 2014) and used to identify miRNAs in *H. contortus*. The reads that mapped to the genome were summarised using featureCounts within the miARma-Seq package. The threshold for miRNA transcription to be recorded was >1 CPM in at least three samples. Targets of miRNAs were predicted using TargetScan (Lewis et al., 2005) and PITA (Kertesz et al., 2007), and the consensus target was recorded.

5.2.4. Protein extraction, LC-MS/MS and proteomic analysis

Proteins were extracted from each of four replicates of L3s, xL3s and L4s (each replicate containing 30,000 individuals). In brief, each larval sample was sonicated (20 kHz) in lysis buffer (8 M urea containing 100 mM triethyl ammonium bicarbonate, pH 8.5) on ice for 20 min, with a 30 s pulse every 30 s. Each sample was supplemented with protease inhibitor cocktail set I (cat no. 539131; Merck, Denmark) and incubated for 1 h at 22 °C. Insoluble debris was removed by centrifugation at 10,000 *g* for 20 min. Protein concentrations were measured using a BCA Protein Assay Kit (cat no. 23227; Thermo Fisher Scientific, USA). Then, samples were subjected to in-solution reduction, alkylation and digestion. In brief, protein aliquots (200 µg) from each sample were reduced with 10 mM tris(2-carboxyethyl)phosphine (TCEP), alkylated with 55 mM iodoacetamide, followed by a double digestion with Lys-C/trypsin Mix (cat no. V5072; Promega, USA) at 37 °C for 16 h. The tryptic samples were acidified with 1.0% (v/v) formic acid and purified using Oasis HLB cartridges (cat no. 186000383; Waters, USA). The processed samples were spiked with calibration peptides (iRT kit, cat no. ki-3002-1; Biognosys, Switzerland) and subjected to LC-MS/MS using a QExactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000).

MS data were analysed by employing Spectronaut software (v.11, Biognosys) using default settings. The spectral library used for 'searching' contained 3350 protein groups (22,721 peptides), generated from in-gel-digested and basic reverse phase-separated peptides from key developmental (i.e., egg, L3, xL3, L4 and adult) stages of *H. contortus* (see Chapter 2). Peptides were accepted based on a false discovery rate (FDR) of < 0.01 at both the peptide and protein levels. The threshold for protein expression to be recorded was ≥ 4 matches in at least four samples.

5.2.5. Comparative analysis

Pairwise comparative analyses were performed based on the identified mRNAs, miRNAs and proteins. To assess the coverage of transcription and expression, the mRNAs and proteins identified in L3, xL3 and L4 stages were compared. To test the differences among these stages, pairwise comparative analyses of transcription and expression were performed. The mRNAs, miRNAs and proteins identified in each stage were compared and displayed using the R package VennDiagram. The transcription or expression levels of the mRNAs, miRNAs and proteins in these stages were subjected to hierarchical cluster analysis using hclust (stat.ethz.ch/R-manual/R-devel/library/stats/html/hclust.html) and illustrated using heatmap2 in the R language (rdocumentation.org/packages/gplots/versions/3.0.1/topics/heatmap.2).

Differentially transcribed mRNAs and miRNAs were identified by pairwise comparison among developmental stages using the limma (Ritchie et al., 2015), Glimma (Su et al., 2017) and edgeR (Robinson et al., 2010) packages (Law et al., 2016) employing a fold change (FC) threshold of > 2, and an FDR of < 0.05. Differentially expressed proteins were identified using the program Spectronaut (v.11, Biognosys) using a threshold of FC of > 2 and FDR of < 0.05. To assess the concordance between mRNA and protein levels, Spearman's correlation analyses of log2-transformed data (CPM for mRNAs; relative abundance for proteins), and log2-transformed FC were performed or calculated using Prism (v.7.0, GraphPad). To evaluate the relationships between miRNAs and their predicted targets, Spearman's correlation and Hoeffding's D measure analyses of log2-transformed data (CPM for mRNAs and miRNAs; relative abundance for proteins)

were conducted using miRLAB (in an R language environment) (Le et al., 2015). The statistical significance of correlation analyses was set at $P < 0.05$.

5.2.6. Functional annotation and integration

Biological functions were assigned to the differentially transcribed mRNAs and differentially expressed proteins as well as targets of differentially transcribed miRNAs based on the Reactome (Vastrik et al., 2007) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Huntley et al., 2016; Kanehisa et al., 2016). Reactome pathway annotation was performed by BLAST (E-value of $< 10^{-5}$) using *Caenorhabditis elegans* orthologs, which were subsequently assigned to pathways using ReactomePA (in an R language environment) (Yu and He, 2016). The enriched Reactome pathway annotations were defined using a threshold of FDR of < 0.05 (Beyer-Hardwick method). By contrast, KEGG pathway annotation was conducted based on KEGG BLAST hits (E-value: $< 10^{-5}$) and corresponding KEGG Orthology (KO) terms (Mao et al. 2005); KO terms were then assigned to KEGG pathways and KEGG BRITE orthologous protein families by mapping KO terms to KEGG Orthology Based Annotation System (KOBAS) database (Xie et al. 2011). Enriched KEGG pathways were identified using a cut-off of $P < 0.01$ (Fisher's Exact test). KEGG functional enrichments of differentially transcribed mRNAs and miRNAs as well as differentially expressed proteins were integrated and displayed using the program FuncTree (Uchiyama et al., 2015).

5.3. Results

5.3.1. Transcriptome, miRNAome and proteome of L3s, xL3s and L4s of H. contortus

Three datasets (i.e., transcriptome, miRNAome and proteome) were produced for each L3, xL3 and L4 of *H. contortus*. For the transcriptome, a total of 9754 mRNA transcripts were identified amongst > 700 million pair-end reads for all three developmental stages (Supplementary Table S5.1). The numbers of mRNA transcripts detected in the L3, xL3 and L4 stages were 9492, 9581 and 9487, respectively (Supplementary Table S5.1). Small RNA libraries yielded > 400 million reads, with 88 miRNAs identified (Supplementary

Table S5.2), of which *hco-miR-34* and *hco-miR-100* were recorded for the first time in *H. contortus*. Similar numbers of miRNAs were identified in L3 (*n* = 86), xL3 (*n* = 87) and L4 (*n* = 84) (Supplementary Table S5.2). Based on the prediction of 11,783 peptides, the total proteome representing L3, xL3 and L4 was inferred to contain 1591 proteins (Supplementary Table S5.3). The numbers of proteins identified in each of the three stages were 1302, 1520 and 1578, respectively (Supplementary Table S5.3).

5.3.2. Transcription and expression of genes among developmental stages

Hierarchical cluster analyses resulted in a division of each transcriptomic (mRNA and miRNA) and proteomic dataset into three distinct groups corresponding to developmental stage (L3, xL3 or L4) of *H. contortus* (Fig. 5.1A-C). The numbers of mRNAs, miRNAs and proteins transcribed/expressed in the three larval stages (L3, xL3 and L4) are shown in Venn diagrams (Fig. 5.1D-F). Here, most mRNAs (*n* = 9092), miRNAs (*n* = 82) or proteins (*n* = 1278) were shared by all larval stages studied, and small numbers (≤ 59) of transcripts or proteins were unique to particular stages.

Specifically, 35 mRNAs were transcribed exclusively in L3s, including those of G protein-coupled receptor (GPCR) domain-containing, zinc finger domain-containing, sperm coating protein (SCP) extracellular-domain or collagen triple helix repeat domain-containing protein-coding genes (Fig. 5.1D); one miRNA (i.e., *hco-miR-34*) was transcribed exclusively in xL3s (Fig. 5.1E); and 56 proteins, including heat shock proteins, peptidases, intestinal prolyl carboxypeptidases, cuticle collagen triple helix repeat domain-containing protein, SCP extracellular domain-containing protein and galectin, were expressed exclusively in L4s (Fig. 5.1F). Pairwise comparisons identified mRNAs that were differentially transcribed between L3 and xL3 (*n* = 1151), between xL3 and L4 (*n* = 1428) and between L3 and L4 (*n* = 2756) stages (Supplementary Table S5.4). Pairwise comparative analysis identified miRNAs that were differentially transcribed between L3 and xL3 (i.e., *hco-miR-34* and *-5976*), between xL3 and L4 (i.e., *hco-miR-34*, *-252*, *-5888*, *-5901*, *-5939* and *-5976*), and between L3 and L4 (i.e., *hco-miR-252*, *-5888*, *-5901*, *-5932*, *-5939* and *-5976*) stages (Supplementary Table S5.5). Pairwise comparative analysis also identified proteins that were differentially expressed between

L3 and xL3 (n = 62), between xL3 and L4 (n = 126), and between L3 and L4 (n = 238) stages (Supplementary Table S5.6).

5.3.3. Relationship between transcription and protein expression

In total, 16.1% of transcripts within the combined transcriptome of L3, xL3 and L4 stages of *H. contortus* represented expressed proteins. Specifically, proteins were identified for 1276, 1483 and 1531 mRNA transcripts in L3, xL3 and L4, respectively (Supplementary Table S5.7), although there was no transcriptomic evidence for 18 proteins (Supplementary Table S5.7).

Levels of mRNA transcription and protein expression were positively correlated in each L3, xL3 and L4 (r = 0.39, 0.44 and 0.43), with a statistical significance of $P < 0.0001$ (Fig. 5.2A-C). Changes in mRNA transcription and protein expression between L3 and xL3 were negatively correlated (n = 1098, r = -0.03, $P > 0.05$), whereas changes between xL3 and L4 (n = 1363, r = 0.20, $P < 0.0001$) and between L3 and L4 (n = 1015, r = 0.30, $P < 0.0001$) stages were positively correlated (Fig. 5.2D-F). Specifically, the correlations in the differential transcription and expression (FC > 2 and FDR < 0.05) between L3 and xL3 (n = 9), between xL3 and L4 (n = 33) and between L3 and L4 (n = 91) stages were r = -0.55 ($P > 0.05$), 0.30 ($P > 0.05$) and 0.31 ($P < 0.01$), respectively (Fig. 5.2D-F).

5.3.4. Association between miRNAs and their gene targets

A total of 86 miRNAs were predicted to target 3773 genes based on both seed pairing and site accessibility, and 6686 mRNA-miRNA pairs were inferred (Supplementary Table S5.8). Of the predicted miRNA-mRNA pairs, 45.4% (L3), 45.3% (xL3) and 40.0% (L4) had negative Spearman's correlation coefficients, and 79.3% (L3), 85.5% (xL3) and 88.1% (L4) had negative Hoeffding's D measure scores, respectively (Supplementary Table S5.8). Specifically, 1019 (27.0%) of the predicted targets were expressed as proteins, and similar percentages of proteins had a negative correlation with miRNAs in L3s (44.7%/83.7%), xL3 (44.6%/96.6%) and L4s (46.8%/99.6%) (Supplementary Table S5.9).

For predicted mRNA-miRNA pairs, changes in mRNA transcription and protein expression were compared in a pairwise manner between larval stages; there was a positive correlation in the level of transcription or protein expression between L3 and xL3 (n = 411, r = 0.03, P > 0.05), between xL3 and L4 (n = 538, r = 0.21, P < 0.0001), and between L3 and L4 (n = 430, r = 0.29, P < 0.0001). By contrast, there was a negative correlation in differential transcription and expression (FC > 2 and FDR < 0.05) between L3 and xL3 (n = 14, r = -0.60, P < 0.05), between xL3 and L4 (n = 16, -0.50, P > 0.05), and between L3 and L4 (n = 16, r = -0.54, P < 0.05) stages (Fig. 5.2G-I).

5.3.5. Biological pathway analysis in different developmental stages

Two approaches were employed to explore the enriched biological functions of the differentially transcribed mRNAs and miRNAs as well as differentially expressed proteins.

First, the Reactome pathway annotation linked differentially transcribed mRNAs and proteins to multiple biological pathways. In L3s, enriched pathways related to the metabolism of fatty acid, triacylglycerol, ketone body, amino acids and derivatives as well as the citric acid (TCA) cycle (Fig. 5.3A); in xL3s, they related to the metabolism of amino acids and derivatives, the TCA cycle and HSF1-dependent transactivation (Fig. 5.3B); in L4s, they related exclusively to the metabolism of amino acids and derivatives (Fig. 5.3C). No functional enrichment was identified for the predicted targets of differentially transcribed miRNAs.

Second, KEGG pathway enrichment analysis revealed that the differentially transcribed and/or expressed genes were associated with biological categories including environmental information processing, genetic information processing, organismal systems and metabolism as well as diseases (Supplementary Tables S5.10-5.12). In L3s, highly transcribed (mRNA) and expressed genes related to carbohydrate metabolism (TCA cycle), lipid metabolism (fatty acid degradation), xenobiotic biodegradation and metabolism, and energy metabolism (oxidation phosphorylation) and diseases (e.g., neurodegenerative, endocrine and/or metabolic processes) (Fig. 5.4A); highly transcribed protein-coding (exclusively) genes related to processes including nucleotide (purine) metabolism, steroid hormone biosynthesis, signalling molecules and interactions

(neuroactive ligand-receptor interaction), and digestive and nervous systems. In xL3s, highly transcribed protein-coding genes were predominantly associated with carbohydrate metabolism (TCA cycle), lipid metabolism (fatty acid degradation and steroid hormone biosynthesis), and xenobiotic biodegradation and metabolism, whereas high miRNA transcription was linked to signal transduction (Rap1 signalling pathway) and endocrine system (peroxisome proliferator-activated receptors [PPAR] signalling) (Fig. 5.4B). In L4s, highly mRNA-transcribed and expressed genes were linked predominantly to transcription, translation, protein folding, sorting and degradation (protein processing in endoplasmic reticulum), amino acid metabolism and the digestive system (protein digestion and absorption) (Fig. 5.4C). In addition, highly transcribed miRNAs (n = 6) related to genes enriched for the Rap1 signalling pathway and the immune system, but the mRNA transcription and protein expression of these genes was low (Fig. 5.4B). Similarly, highly transcribed miRNAs (n = 6) related to genes involved in signalling molecules and interactions, but this was not the case for mRNAs (Fig. 5.4C).

5.4. Discussion

Using transcriptomic, miRNA and proteomic datasets, we explored molecular alterations in the developmental switch from L3 to L4 of *H. contortus* in vitro. Key changes were seen in mRNA, miRNA and protein profiles, and plausible relationships were established between the transcription (mRNA and/or miRNA) and protein expression.

Qualitative and quantitative analyses performed in this study identified substantial molecular changes in the developmental switch of *H. contortus*. The molecular changes were reflected by the specific transcription and expression between developmental stages. Although most mRNAs, miRNAs and proteins detected were shared by all three larval stages, exclusive transcription or expression indicated specific biological features for individual stages. In particular, the specific transcription of GPCR domain-containing and zinc finger domain-containing protein-coding genes suggested important chemosensory activities in the L3 stage, as the roles of these molecules in signal transduction and transcriptional regulation have been reported in *Caenorhabditis elegans* (see Clarke and Berg, 1998; Bargmann et al., 2006; Kim et al., 2009; Procko et al., 2012). Similarly, the

exclusive expression in L4 of peptidases, SCP and galectin, which associate with protein digestion and immune responses, suggest features characteristic of a parasitic stage, such as blood feeding and parasite-host interactions (Laing et al., 2013; Schwarz et al., 2013). Molecular changes were also reflected in different miRNA, mRNA and protein levels in the developmental stages of *H. contortus*. Although only seven miRNAs with significant transcriptional changes among the three developmental stages were identified, substantial changes in transcription and expression were detected for thousands of mRNAs and hundreds of proteins, respectively. In particular, the marked differential transcription and expression between the L3 and L4 stages of *H. contortus* indicated significant alterations during the in vitro developmental transition from the free-living to the parasitic stage of this parasite.

To further understand the molecular alterations, we tested the concordance between transcriptome and proteome. For 16.1% of total mRNA transcription, a positive correlation (r = 0.39 to 0.44) with protein expression in all three developmental stages of *H. contortus* was observed. In contrast, negative and positive correlations (r = -0.55 to 0.31) were seen for differential mRNA transcripts and proteins between developmental stages. These results are consistent with previous studies of eukaryotes (Csárdi et al., 2015; Edfors et al., 2016; Lahtvee et al., 2017), and might relate to technical aspects in protein discovery, but are likely explained by a predominance of post-transcriptional regulation (Csárdi et al., 2015). For example, the most studied post-transcriptional regulators, miRNAs, play key roles in translational repression and mRNA degradation (Kim, 2005; Filipowicz et al., 2008; Bartel, 2009; Morris and Mattick, 2014) and would explain the tightly controlled expression seen here in *H. contortus.*

Therefore, to understand whether miRNAs associate with the discrepancy between transcriptome and proteome, we explored the relationships among miRNAs, mRNAs and proteins. First, targets of miRNAs were predicted using two different algorithms, considering both base-pairing (TargetScan) and site accessibility (PITA), to increase the accuracy of prediction (Lewis et al., 2005; Kertesz et al., 2007). These prediction tools have been applied to other nematodes such as *Caenorhabditis elegans* and *Trichuris suis*, and have produced convincing results (Jan et al., 2011; Jex et al., 2014). Then, we used two independent parameters (using Spearman's correlation and Hoeffding's D measure; Fujita et al., 2009) to assess the reliability of target prediction. The reliability of the

predictions was indicated by a strong negative correlation between levels of miRNAs and mRNAs (40.0%-88.1%), and between levels of miRNAs and proteins (44.6%-99.6%). The high correlation between miRNAs and proteins indicate substantial translational repression. This proposal is supported by the target predictions in this study, which were performed against 3'-untranslated region (UTR) sequences, and by limited protein expression of the predicted targets (27.0%). Furthermore, the consistent negative correlation (r = -0.60 to -0.50) between the differential mRNA transcription and differential protein expression indicates substantial post-transcriptional regulation.

Since there were substantial differences in the transcription of mRNA and miRNA as well as protein expression between the developmental stages of *H. contortus*, the next step was to clarify the biological meaning of these differences. Based on Reactome pathway analysis (for *Caenorhabditis elegans*), the enriched pathways inferred related to carbohydrate and fatty acid metabolism in the L3 stage, and the metabolism of amino acids and derivatives in the L4 stage. However, although *H. contortus* is closely related to *Caenorhabditis elegans* (see Blaxter et al., 1998), and the latter has been widely used as a model organism for parasitic nematodes (Politz and Philipp, 1992; Bürglin et al., 1998; Gilleard, 2004), *Caenorhabditis elegans* is a free-living nematode, restricting our ability to annotate molecules linked to parasitic biological processes (Geary and Thompson, 2001; Datu et al., 2008; Viney, 2017). This aspect was reflected in the limited number of Reactome pathway annotations, particularly in the parasitic L4 stage of *H. contortus*. Nevertheless, to address this issue, we also employed the KEGG database to assign biological meaning to the molecular changes, and, indeed, more enriched biological categories were predicted for differential mRNAs, miRNAs and proteins using KEGG than for the Reactome pathway analysis.

Based on the Reactome and KEGG pathway analyses, enriched fatty acid and carbohydrate degradation, TCA cycle, oxidation phosphorylation and neuroactive ligand-receptor interaction in L3s indicated the specific features (e.g., inability to feed, aerobic metabolism and active chemosensation) for this free-living stage (Nikolaou and Gasser, 2006; Cantacessi et al., 2010; Wang et al., 2016). These features also linked to the annotations of neural, endocrine and metabolic diseases, suggesting a dysregulated physiological and biochemical status, which accords with dauer in *Caenorhabditis elegans* (cf. Hu et al., 2007). The similarity between the L3 stage of some parasitic

nematodes and the dauer stage of *Caenorhabditis elegans* has been extensively discussed (Beall and Pearce, 2002; Ogawa et al., 2009; Crook, 2014; Lok, 2016), and it has been proposed that the dauer stage is a pre-adaptation to parasitism (cf. Crook, 2014).

By contrast, down-regulated or suppressed pathways in the xL3 stage, such as fatty acid degradation, steroid hormone biosynthesis, PPAR signalling, hormone signal transduction and neuroactive ligand-receptor interaction, indicated switches in metabolism and changes in signalling in the developmental transition from L3 to L4 in vitro (cf. Bento et al., 2010; Lee and Schroeder, 2012; Lok, 2016; Butcher, 2017; Watts and Ristow, 2017). Additionally, the enriched pathways in the L4 stage, linked to amino acid metabolism, transcription, translation, protein processing in endoplasmic reticulum and pathogenesis, indicate biological processes for rapid development and parasitic features (e.g., blood feeding in vivo) (Nikolaou and Gasser, 2006; Laing et al., 2013; Schwarz et al., 2013). Notably, the present, integrated analysis showed that not all transcriptional enrichments translated into functional (protein) up-regulations. While such a discrepancy might be explained by limited coverage of the proteome, this is highly unlikely to be the case here. The most plausible explanation is a tight post-transcriptional regulation of gene expression. Taken together, the present findings reveal that the significant biological changes observed during the development from the L3 to the L4 stage of *H. contortus* in vitro relate to: (i) environmental information processing converting into genetic information processing; (ii) energy production switching from carbohydrate and lipid degradation to protein metabolism; and (iii) free-living molecular features adapting to parasitic traits.

The adaptation of *H. contortus* to its environment during its developmental transition in vitro appears to be tightly regulated by miRNA networks. Specifically, the enriched HSF1-dependent transactivation and xenobiotic biodegradation and metabolism pathways in the xL3 stage indicate stress responses (Dayalan Naidu and Dinkova-Kostova, 2017), which might be caused by exogenous stimuli such as NaClO, CO_2 and/or high temperature during artificial exsheathment; under this condition, the exclusive, high transcription of *hco-miR-34* in the xL3 stage strongly suggests a functional role in stress responses. The upregulated transcription of *miR-34* has also been reported in the dauer stage of *Caenorhabditis elegans* and the infective stage of *Strongyloides ratti* (see Ahmed et al., 2013; Isik et al., 2016), and might be involved in the morphological and metabolic

adaptation through interactions with *daf-2* and *daf-16*, in response to environmental stresses (Isik et al., 2016). Additionally, the high transcription of *hco-miR-252* in the L4 stage might associate with pathogen-related responses, which accords with previous studies of *Caenorhabditis elegans* (see Kudlow et al., 2012; Sun et al., 2016). However, the functional roles of *hco-miR-34* and *hco-miR-252* need to be explored to understand why the Rap1 signalling pathway is suppressed in both xL3 and L4 stages, and what the downstream regulatory networks are. Moreover, *hco-miR-5888, -5901, -5932* and *-5976* are highly transcribed in parasitic stages, but nothing is yet known about the functional roles of these small RNAs in *H. contortus* (see Winter et al., 2012). These "novel" miRNAs might play crucial roles in the development of this parasite, and thus warrant detailed investigation.

In conclusion, the integrated transcriptomic, proteomic and bioinformatic investigation reported here has given detailed insights into molecular alterations during the developmental transition from the free-living to parasitic larval stages of *H. contortus* in vitro. Dominant post-transcriptional regulations were inferred, and miRNAs likely play critical roles during the in vitro developmental transition. This transition involves a range of biological changes associated with metabolism, signal transduction and genetic information processing. Specifically, *hco-miR-34* and *hco-miR-252* appear to play key roles in the molecular alterations, particularly in stress responses and environmental adaptation. Taken together, these findings significantly enhance our understanding of the developmental biology of this important parasite at the molecular level under well-defined conditions outside of the host. Future work should compare the molecular developmental transitions in vitro with those that occur in vivo within the host animal.

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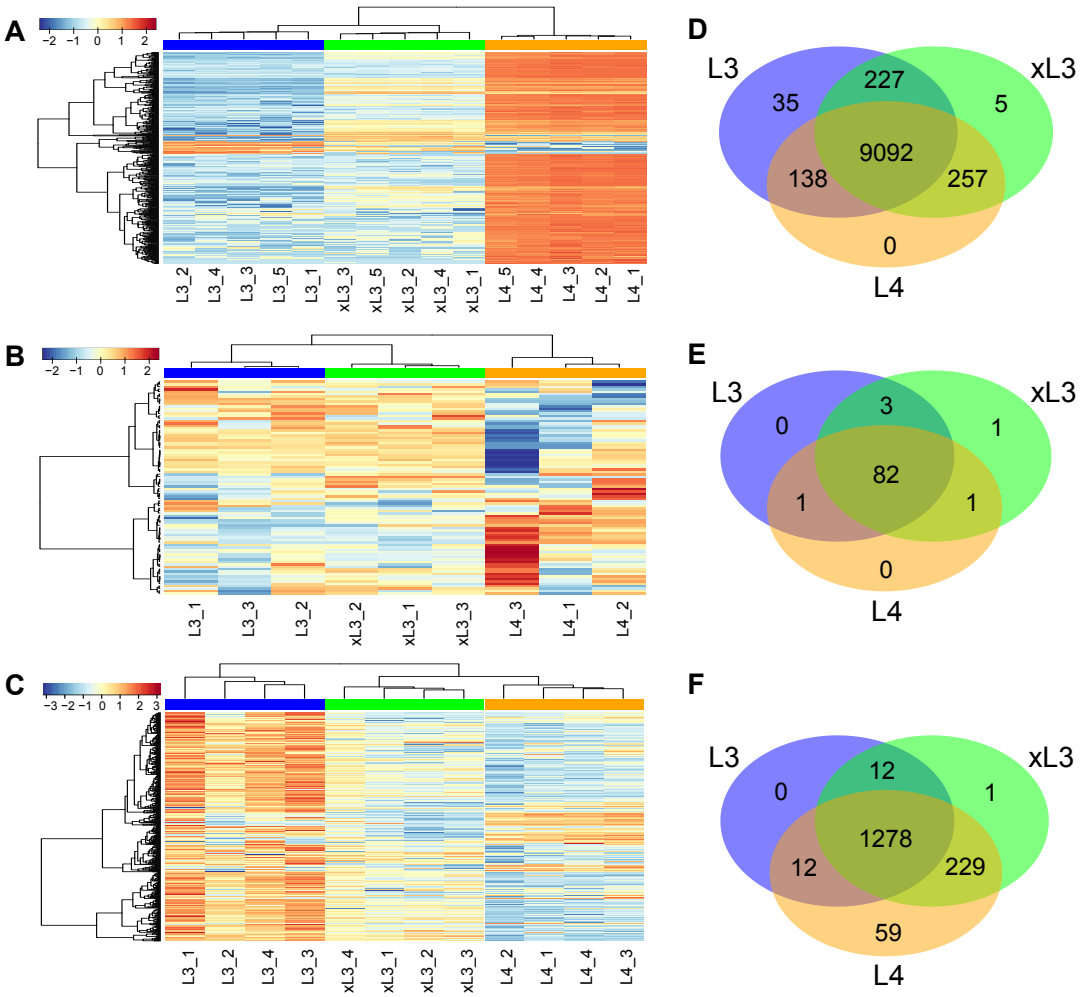


Fig. 5.1. Stage-specific transcriptome, microRNAome and proteome of *Haemonchus contortus*. Heat maps display transcription or expression profiles (log_2-transformed counts per million (CPM) or abundance) of (A) mRNAs (top 500), (B) miRNAs (top 80) and (C) proteins (top 500) representing the L3, exsheathed L3 (xL3) and L4 stages of *H. contortus.* Profiles for L3, xL3 and L4 clustered into three groups (blue, green and orange bars). Venn diagrams show the numbers of (D) mRNAs, (E) miRNAs and (F) proteins unique to the L3 (blue), xL3 (green) or L4 (orange) of *H. contortus*, and shared by two or three of these developmental stages (intersections).

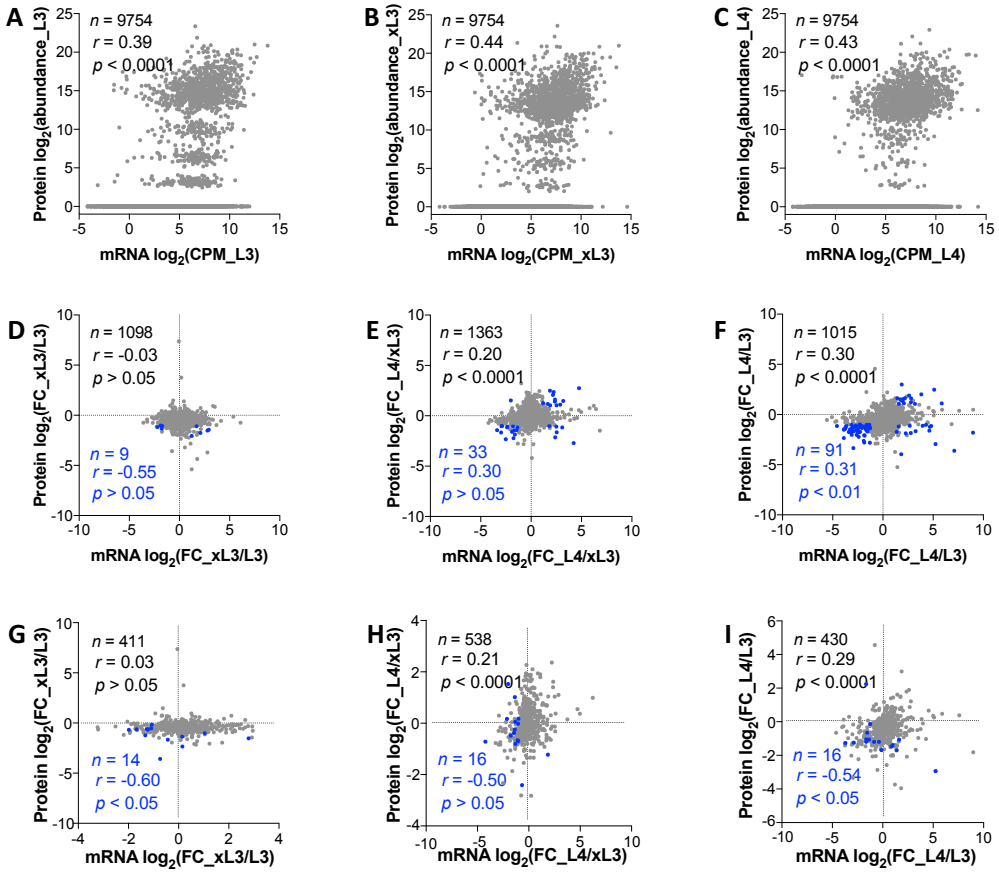


Fig. 5.2. Relationships between mRNA transcription and protein expression levels in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus*. Levels of mRNAs (log_2-transformed counts per million, CPM) are related to protein levels (log_2 abundance). Correlations of the mRNA and protein levels are shown for (A) L3, (B) xL3 and (C) L4 stages; correlations of the changes (log_2-transformed fold change, FC) in mRNA and protein levels between (D) L3 and xL3, (E) xL3 and L4, and (F) L3 and L4 stages are shown; correlations of the changes in mRNA and protein levels of miRNA targets between (G) L3 and xL3, (H) xL3 and L4 and (I) L3 and L4 stages are displayed. The number of mRNA-protein pairs (*n*), correlation coefficients (*r*) and significance (*P*) are indicated. Dots and values for differentially transcribed/expressed molecules (*n*, *r* and *P*) are highlighted in blue.

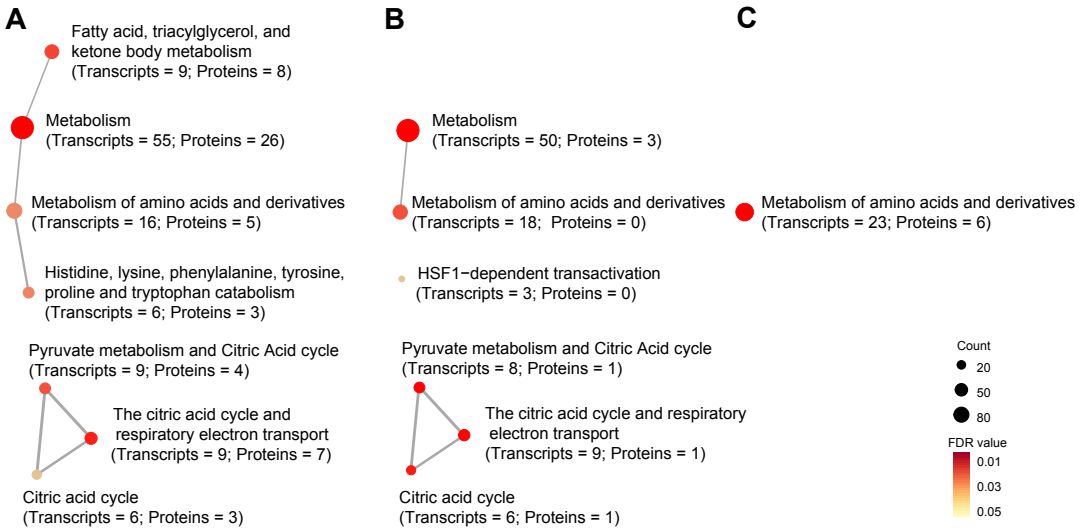


Fig. 5.3. Enriched pathways (Reactome) of differential transcription and expression in *Haemonchus contortus*. Enriched pathways for differentially transcribed mRNAs and expressed proteins in the (A) L3, (B) exsheathed L3 (xL3) and (C) L4 stages of *H. contortus*. Dot sizes indicate counts of significantly highly (fold change (FC) of > 2; false discovery rate (FDR) of < 0.05) transcribed mRNAs and expressed proteins. The numbers of differentially transcribed mRNAs and/or expressed proteins mapped to Reactome pathways are indicated. Statistical significance (false discovery rate, FDR) of enrichment is indicated in colour.

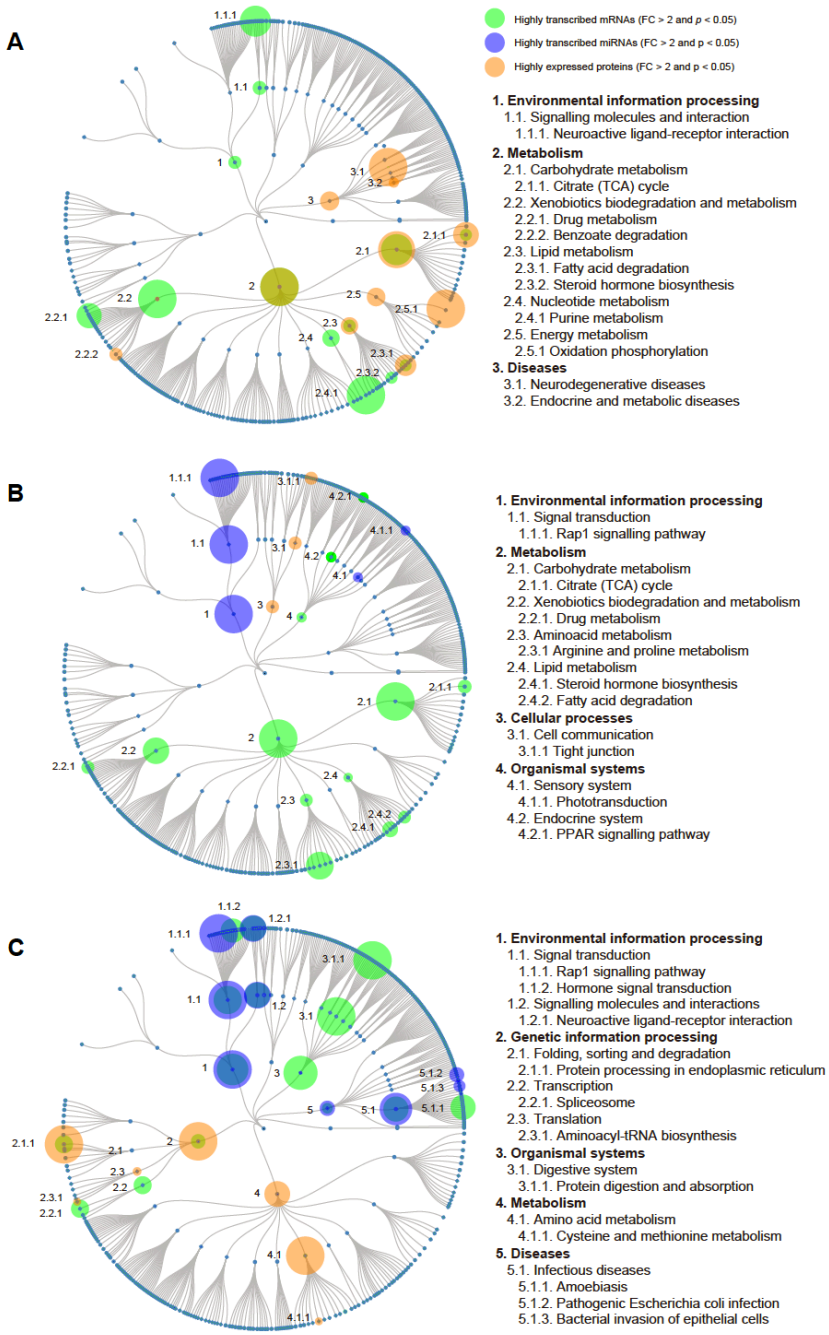


Fig. 5.4. Enriched biological functions (via Kyoto Encyclopedia of Genes and Genomes, KEGG) of differential transcription and expression in *Haemonchus contortus*. Enriched biological processes and associated pathways of differential mRNAs, microRNAs and proteins in the (A) L3, (B) exsheathed L3 (xL3) and (C) L4 stages of *H. contortus*. Annotations are illustrated by hierarchical biological categories, biological processes and KEGG pathways. Enriched annotations of highly transcribed mRNAs and miRNAs (fold change (FC) of > 2; false discovery rate (FDR) of < 0.05), or highly expressed proteins (fold change (FC) of > 2; false discovery rate (FDR) of < 0.05) are indicated by green, orange and purple dots, respectively, and listed. Dot sizes indicate counts of significantly highly transcribed mRNAs and/or expressed proteins (see also Supplementary Tables S5.10-5.12).

Supplementary Table S5.1. Transcription profiles of mRNA in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.2. Transcription profiles of microRNA in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.3. Expression profiles of protein in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.4. Pairwise comparative analysis of mRNAs between L3 and exsheathed L3 (xL3), between xL3 and L4 and between L3 and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.5. Pairwise comparative analysis of microRNAs (miRNA) between L3 and exsheathed L3 (xL3), between xL3 and L4 and between L3 and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.6. Pairwise comparative analysis of proteins between L3 and exsheathed L3 (xL3), between xL3 and L4 and between L3 and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.7. Correlation analysis of mRNA and protein levels in and the changes in mRNA and protein levels among the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.8. Spearman's correlation and Hoeffding's D measure analyses of microRNA (miRNA) and mRNA levels in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.9. Spearman's correlation and Hoeffding's D measure analyses of microRNA (miRNA) and protein levels among the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.10. Enriched biological categories, biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways representing differentially transcribed mRNAs in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.11. Enriched biological categories, biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially transcribed microRNAs (miRNA) in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

Supplementary Table S5.12. Enriched biological categories, biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed proteins in the L3, exsheathed L3 (xL3) and L4 stages of *Haemonchus contortus.*

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Chapter 6. Proposed dauer signalling pathways model for *Haemonchus contortus*

Abstract

Signalling pathways have been extensively investigated in the free-living nematode *Caenorhabditis elegans*, but very little is known about these pathways in parasitic nematodes. Here, we constructed a model for the dauer-associated signalling pathways in an economically significant parasitic worm, *Haemonchus contortus*. Guided by the information available for *Caenorhabditis elegans*, we used extensive genomic and transcriptomic data sets to infer gene homologues in the dauer-associated pathways, explore developmental transcriptomic and proteomic profiles in *H. contortus* and study selected molecular structures. The canonical cyclic guanosine monophosphate (cGMP), transforming growth factor-β (TGF-β), insulin/insulin-like growth factor 1 (IGF-1) and steroid hormone signalling pathways of *H. contortus* were inferred to represent a total of 61 gene homologues. Compared with *Caenorhabditis elegans*, *H. contortus* has a reduced set of genes encoding insulin-like peptides (INS), implying evolutionary divergences between the parasitic and free-living nematodes. Similar transcription profiles were found for all gene homologues between the infective stage of *H. contortus* and dauer stage of *Caenorhabditis elegans*. High transcriptional levels for genes encoding G protein-coupled receptors (GPCRs), TGF-β, INS (e.g., *ins-1*, *ins-17* and *ins-18*) and transcriptional factors (e.g., *daf-16*) in the infective L3 stage of *H. contortus* were suggestive of critical functional roles in this stage. Conspicuous protein expression patterns and extensive phosphorylation of some components of these pathways suggested marked post-translational modifications also in the L3 stage. The high structural similarity in the DAF-12 ligand binding domain among nematodes indicated functional conservation in steroid (i.e., dafachronic acids) signalling linked to worm development. Taken together, this pathway model provides a basis to explore hypotheses regarding biological processes and regulatory mechanisms (via particular phosphorylation events and/or lipids) associated with the development of *H. contortus* and related nematodes as well as parasite-host cross talk, which could aid the discovery of new therapeutic targets.

6.1. Introduction

Signal transduction is a process by which physical or chemical impulses are transmitted through a cell via a series of molecular events (Gammons and Bienz, 2017; Gushchin and Gordeliy, 2018). Many signalling pathways involve the binding of molecules (called ligands) to receptors that initiate or activate processes within cells. Usually, ligand binding leads to conformational alteration(s) in a receptor, which triggers a chain of biochemical events that regulate processes such as transcription and translation, post-translational or conformational alterations of proteins and/or the location of expression (Deribe et al., 2010; Manning and Toker, 2017; Hilger et al., 2018).

Signalling pathways have been extensively investigated in the free-living nematode *Caenorhabditis elegans* (see Greenwald, 2005; Huang and Sternberg, 2006), which is arguably the best-characterised and most tractable metazoan model organism (Corsi et al., 2015; Lee et al., 2017). Particularly well studied are pathways governing/regulating growth, development, reproduction and lifespan (Gerisch and Antebi, 2004; Kenyon, 2005; Fielenbach and Antebi, 2007; Hammell et al., 2009; Viney and Harvey, 2017), including those of the canonical "dauer signalling" complex (Bargmann, 2006; Hu, 2007; Gumienny and Savage-Dunn, 2013; Murphy and Hu, 2013; Antebi, 2015). Here, environmental signals are received by chemosensory neurons through the cyclic guanosine monophosphate (cGMP) signalling pathway (Bargmann, 2006), then transduced via the transforming growth factor-β (TGF-β) signalling and insulin/insulin-like growth factor 1 (IGF-1) signalling pathways in neuroendocrine tissues (Gumienny and Savage-Dunn, 2013; Murphy and Hu, 2013), and converted to steroid hormone signals (e.g., dafachronic acids, DAs) to regulate cellular processes critical to development, reproduction and lifespan (Gerisch and Antebi, 2004; Kenyon, 2005; Lee and Schroeder, 2012; Antebi, 2015). Although homologous signalling pathways have been proposed or partially described for parasitic nematodes (Tissenbaum et al., 2000; Beall and Pearce, 2002; Viney, 2005; Crook, 2014; Lok, 2016), knowledge is incomplete and functional information mostly lacking. Clearly, the availability of comprehensive genomic and transcriptomic data sets for nematodes of the order Strongylida (parasite.wormbase.org) (see Chapter 5; Laing et al., 2013; Schwarz et al., 2013; Preston et al., 2017; Doyle et al., 2018), which belong to the same evolutionary clade as

Caenorhabditis elegans, provides a unique opportunity and foundation to explore the dauer signalling pathways in socioeconomically important representatives of this order.

Haemonchus contortus (barber's pole worm) is one such representative, arguably one of the most important gastrointestinal nematodes of ruminants worldwide (Gasser and von Samson-Himmelstjerna, 2016). This worm has a complex life cycle from the egg to the adult stage, under variable environmental and host conditions (Veglia, 1915). Specifically, the third-stage larvae (L3s) of *H. contortus* survive in the environment and will not develop to the next stage until activated by cues in the gut of the host animal, similarly the fourth-stage larvae (L4s) may arrest in development within the host in response to seasonal and/or host factors (Gibbs, 1986; Besier et al., 2016; Nisbet et al., 2016). Although previous studies have explored aspects of TGF-β and IGF-1 signalling pathways in *H. contortus* and investigated the functionality of selected molecules, including *Hc-hsp-90*, *Hc-daf-16* and *Hc-daf-2* (e.g., Beall and Pearce, 2002; Nikolaou and Gasser, 2006; Gillan et al., 2009; Hu et al., 2010; Gilabert et al., 2016; Li et al., 2014a, 2014b, 2016; Mohandas et al., 2016), there are still significant gaps in our knowledge and understanding of the signalling pathways linked to dauer in this species.

Here, employing extensive, publicly available genomic and transcriptomic data sets for *H. contortus*, and guided by molecular and functional resources available for *Caenorhabditis elegans*, we infer the gene homologues representing four inter-connected (dauer-associated) signalling pathways in *H. contortus* and explore developmental transcriptomic, proteomic and phosphoproteomic profiles for pathway components. This pathway reconstruction and validation should provide a basis for functional studies of dauer signalling in *H. contortus* and related nematodes, which might facilitate the discovery of new anthelmintic targets.

6.2. Materials and methods

6.2.1. Identifying Caenorhabditis elegans gene homologues in H. contortus

A list of all genes (n = 102) and gene products (n = 182) representing the cGMP, TGF-β and IGF-1 signalling pathways as well as the steroid hormone pathway in *Caenorhabditis elegans* was established based on published information (e.g., Hu, 2007;

Stoltzfus et al., 2012; Gilabert et al., 2016) (Supplementary Table S6.1). The gene and protein sequences, their accession numbers and transcriptomic data were obtained from WormBase (v.WS261). Homologues of these genes were identified by searching (tblastn; *e*-value: ≤ 10^{-5}) the *Caenorhabditis elegans* protein sequences against gene predictions from the latest, published genome and the transcriptomes of *H. contortus* (see Chapter 5; Schwarz et al., 2013; Preston et al., 2017; Doyle et al., 2018). The *Caenorhabditis elegans* protein sequences were also searched against the *H. contortus* genome using BLAT v.34 (Kent, 2002) to identify homologues. The gene sequences identified were compared (blastx; *e*-value: ≤ 10^{-5}) with *Caenorhabditis elegans* proteins (PRJNA13758.WS261) to cross-verify their identity.

6.2.2. Gene curation and structural modelling

Genes and transcripts were curated using a recently established method (Stroehlein et al., 2018). In brief, the sequences inferred to represent homologues were mapped to the genome assembly of *H. contortus* using the program BLAT v.34; mapping was displayed using the Integrated Genome Viewer v.2.4.4 (IGV). The mapped transcripts were reassembled using the program CAP3 (Huang and Madan, 1999) for possible extensions. The reassembled transcript sequences were mapped to the genome assembly of *H. contortus* (see Doyle et al., 2018), and the corresponding coding DNA sequences (CDS) in the genome were refined using the "coding2genome" model in the program Exonerate v.2.2.0 (Slater and Birney, 2005). The sequences of curated genes were cross-checked with those of complementary DNAs (cDNAs) for *Hc-daf-16*, *Hc-daf-2* and *Hc-pdk-1* (Hu et al., 2010; Li et al., 2014b, 2016). Subsequently, open reading frames (ORFs) were predicted using the program ORF finder (Rombel et al., 2002), and structural and functional domains identified using InterProScan v.61.0 (Zdobnov and Apweiler, 2001; Jones et al., 2014). Pairwise comparisons of inferred amino acid sequences were performed using the program MAFFT v.7.309 (Katoh et al., 2002).

Structural modelling was conducted for a nuclear hormone receptor (DAF-12) using the program I-TASSER (Yang et al., 2015), following the alignment of amino acid sequence data in MAFFT v.7.309. The alignment was viewed in MView v.1.62 (Brown et al., 1998). Models were displayed and compared with available crystal structures

(Wang et al., 2009) using UCSF Chimera v.1.12 (Pettersen et al., 2004), and structural similarities between query and template sequences were measured using TM-score and root-mean-square deviation (RMSD) (Yang et al., 2015). Biological functions (Gene Ontology, GO) of the modelled protein domain were inferred based on structural similarity.

6.2.3. Transcription analysis

RNA-seq reads (paired-end) from individual developmental stages/sexes of the nematode were mapped to individual curated CDS in the genome using Bowtie v.2.1.0 within the software package RSEM v.1.2.11 (Li and Dewey, 2011; Langmead and Salzberg, 2012). At least 10 reads needed to map to a CDS for transcription to be recorded. Transcription levels of messenger RNAs (mRNAs) were recorded in fragments per kilobase per million mapped reads (FPKM). For individual genes of individual developmental stages of *H. contortus*, transcription levels were displayed in a heat map using heatmap.2 in an R-language environment (v.3.5.1).

6.2.4. Protein analyses

Proteomic analysis of *H. contortus* was conducted using an established protocol (see Chapters 2 and 5). In brief, protein sequences predicted from individual homologous genes were used to search mass spectrometric (MS) data representing the egg, L3, L4 (female and male) and adult (female and male) stages of *H. contortus* using Proteome Discoverer software v.2.0 (Thermo Fisher Scientific). Peptides were identified using a false discovery rate (FDR) cut-off of < 1% at the peptide and protein levels. Peptide intensities were calculated using Spectronaut software v.11 (Biognosys). At least two peptides needed to match a corresponding protein sequence for expression to be recorded. Peptide intensities were employed to infer the expression levels of individual protein homologues in different developmental stages of *H. contortus*. A phosphoproteomic analysis of egg, L3, L4 (female and male) and adult (female and male) stages of *H. contortus* was conducted using an established TiO_2 enrichment protocol (Zielinska et al., 2009; Borchert et al., 2012). Protein sequences encoded by dauer signalling gene

homologues were employed to interrogate the phosphoproteomic data using the Proteome Discoverer software. Phosphopeptides were identified using a FDR cut-off of < 1% at the peptide and protein levels. Phosphorylated proteins were mapped to dauer signalling pathways in *H. contortus*.

6.3. Results

6.3.1. The dauer signalling pathway model

First, we identified the genes (n = 102; Supplementary Table S6.1) representing the cGMP, TGF-β, IGF-1 and steroid hormone signalling pathways in *Caenorhabditis elegans*, and constructed the canonical framework for the dauer signalling pathway model (Fig. 6.1).

Second, we identified all homologues of these genes in *H. contortus*. To do this, we conducted exhaustive homology searches against the genome and combined transcriptome of *H. contortus*, which yielded more than 25,000 sequences in *H. contortus*, from which we predicted genes and transcripts. These genes and transcripts, relating to 61 full-length coding sequences, were curated and assigned names (Supplementary Table S6.2). *Hc-daf-1* was represented by two paralogues (Supplementary Table S6.2). All 61 genes were transcribed, and transcripts had domain sequence-matches with respective amino acid sequences encoded by *Caenorhabditis elegans* genes (Supplementary Table S6.2).

Third, we mapped the 61 genes to the framework constructed for *Caenorhabditis elegans* (Fig. 6.1), providing a model for dauer signalling pathways in *H. contortus*. From this mapping, 60 of the 102 *Caenorhabditis elegans* dauer signalling genes were homologous to the 61 genes in *H. contortus*. However, not detected in the parasitic nematode were genes encoding proteins SRBC-64, SRBC-66, SRG-36 and SRG-37 (G protein-coupled receptors, GPCRs); AKT-2 (serine/threonine kinase), PAR-5 (14-3-3 protein), DAF-28 (insulin), INS-2 to INS-16 and INS-19 to INS-37 (insulin-like peptides); and NCR-2 (membrane permease), inferred or known to be involved in dauer signalling in *Caenorhabditis elegans*.

6.3.2. Signalling cascades

Based on our model for *H. contortus*, we hypothesise the following events in the four components of the signalling cascade:

For cGMP signalling (8 components; Supplementary Table S6.2), environmental cues are sensed by *Hc*-DAF-37 and -DAF-38 (GPCRs) that activate *Hc*-GPA-2 and -3 (GTPase), which initiates cGMP production by *Hc*-DAF-11 (receptor-like guanylyl cyclase) and -DAF-21 (heat shock protein 90), controlling the activity of the transmembrane channels formed by *Hc*-TAX-2 and -TAX-4 (cGMP-gated channel subunits).

For TGF-β signalling (14 components; Supplementary Table S6.2), *Hc*-DAF-7 (TGF-β) is bound by *Hc*-DAF-1 and -DAF-4 (heterotetrameric TGF-β receptor), which activates DAF-8 and DAF-14 (Smad protein complex) that is inhibited by PDP-1 (serine/threonine phosphatase), inhibiting the function of *Hc*-DAF-3 and -DAF-5 (transcription regulator complex) which are regulated by *Hc*-EGL-4 (cGMP-dependent protein kinase), SCD-1 and -2 (receptor tyrosine kinases) and FSN-1 (F-box synaptic protein).

For IGF-1 signalling (26 components; Supplementary Table S6.2), ASNA-1 (ATPase) promotes the secretion of *Hc*-INS-1, -INS-17 and -INS-18 (insulin-like peptides) from chemosensory neurons, which bind to *Hc*-DAF-2 and -IST-1 (receptor tyrosine kinase and substrate) that regulate sequential phosphorylation by *Hc*-AAP-1, -AGE-1 (phosphoinositide 3-kinase), -PITP-1 (phosphatidylinositol transfer protein), -PDK-1 (3-phosphoinositide-dependent kinase), -SGK-1 and -AKT-1 (serine/threonine kinases), regulating the cytoplasmic sequestration of transcription factors *Hc*-HSF-1 (with *Hc*-DDL-1 and heat shock binding protein *Hc*-HSB-1), -SKN-1 and -DAF-16 (with 14-3-3 protein FTT-2). *Hc*-DAF-18 (lipid phosphatase), -PPTR-1 (serine/threonine phosphatase) and -RLE-1 (E3 ubiquitin ligase) regulate the phosphorylation of *Hc*-AGE-1, -AKT-1 and -DAF-16, respectively. The interactors in this signalling pathway also include *Hc*-ACS-19 (acyl-CoA synthetase), -DAF-15 (raptor protein), LDB-1 (LIM Domain Binding protein), -SOD-3 (iron/manganese superoxide dismutase) and a homologue of Y105E8B.9 (glutathione transferase).

For steroid hormone signalling (13 components; Supplementary Table S6.2), extracellular cholesterol and glycolipids are transported into neuroendocrine cells by *Hc*-NCR-1 (membrane permease), and sequentially modified by *Hc*-DAF-36 (Rieske oxygenase), -STRM-1 (methyltransferase), -DHS-16 (dehydrogenase), -DAF-9 (cytochrome P450) and -EMB-8 (NADPH-cytochrome P450 reductase), or directly modified by an *Hc*-HSD (hydroxysteroid dehydrogenase), resulting in the production of DAs that activate *Hc*-DAF-12. In turn, the activation of *Hc*-DAF-12 promotes the production of DAs via *Hc*-DAF-9 in target (hypodermal) cells. The binding of DA or *Hc*-DIN-1 (nucleic acid binding protein) to *Hc*-DAF-12 (nuclear hormone receptor) forms a co-activator or co-repressor complex, regulating downstream *Hc*-GCK-2 (mitogen-activated protein kinase kinase kinase kinase), -LEV-9 (sushi domain-containing protein), -LIT-1 (serine threonine protein kinase) and -UGT-65 (UDP glucuronosyltransferase).

6.3.3. Molecular profiles throughout development

We established the transcription profiles of dauer signalling genes for all key developmental stages of *H. contortus* (egg, the first-larval (L1), second-larval (L2), L3, L4 stages, and female and male adult stages) and compared these profiles with those inferred for homologues in respective stages of *Caenorhabditis elegans*. In both nematode species, each homologous gene was transcribed in at least one developmental stage. Transcription profiles were similar between the L3 stage of *H. contortus* and dauer stage of *Caenorhabditis elegans* (Fig. 6.2). In these developmental stages of the two species, peak transcription levels were detected for genes *daf-37*, *gpa-2, gpa-3, daf-11* and *tax-2* in the cGMP signalling pathway; *daf-14* and *egl-4* in the TGF-β signalling pathway; *ins-1*, *ins-17*, *ins-18*, *ist-1*, *akt-1*, *skn-1* and *daf-16* in the IGF-1 signalling pathway; and *emb-8*, *daf-12* and *din-1* in the steroid hormone signalling pathway (Fig. 6.2; Supplementary Tables S6.1-6.3). By contrast, there were distinct differences in profiles for other genes. Although high transcription levels were recorded for *daf-38* and *tax-4* (cGMP signalling), *daf-7*, *daf-1*, *daf-4*, *daf-3* and *daf-5* (TGF-β signalling), *daf-2* and *pitp-1* (IGF-1 signalling), and *daf-9* and *lev-9* (steroid hormone signalling) in *H. contortus* L3, transcriptional peaks for these genes were found in L1 or L2 stages of *Caenorhabditis elegans* (Fig. 6.2; Supplementary Tables S6.1-6.3). Marked differences

in the transcription of selected genes, including *daf-21*, *daf-1*, *daf-4* and *daf-2*, were found between the two species in the egg, L4 and adult stages (Fig. 6.2).

We also explored the protein expression of the dauer signalling genes in *H. contortus* as well as the phosphorylation of proteins. In total, proteins encoded by 11 signalling genes were detected in the egg, L3, L4 and/or adult stages of the parasite (Supplementary Table S6.4). These genes included *Hc-daf-21* (cGMP signalling), *Hc-egl-4* and *Hc-pdp-1* (TGF-β signalling pathway), *Hc-akt-1*, *Hc-pptr-1*, *Hc-asna-1*, *Hc-ftt-2* and *Hc-sod-3* (IGF-1 signalling), and *Hc-emb-8*, *Hc-din-1* and *Hc-gck-2* (steroid hormone signalling). In these proteins, one to 6 phosphorylation sites was/were detected for *Hc*-DAF-21 (cGMP signalling), *Hc*-AKT-1 and -PPTR-1 (IGF-1 signalling), -DIN-1 and -GCK-2 (steroid hormone signalling) (Supplementary Table S6.4). These five phosphorylated proteins were detected in egg and/or larval stages of *H. contortus*, particularly L3. However, none of them was found to be phosphorylated in female or male adult stages of this species.

6.3.4. DAF-12 - the checkpoint in dauer signalling

As DAF-12 is a determining point in the dauer signalling pathways, we explored its features and conservation. Pairwise comparisons of the inferred DAF-12 protein sequences of *H. contortus* (*Hc*-DAF-12a), *Caenorhabditis elegans* (WormBase ID: CE27585), *Strongyloides stercoralis* (GenBank accession no. AAD37372), *Ancylostoma ceylanicum* (EPB79655) and *Necator americanus* (XP_013307217) revealed sequence identities of 35.5% to 86.3%. High sequence similarities were in the zinc finger (nuclear hormone receptor type) and ligand binding domains (Fig. 6.3A). The three-dimensional models for the DAF-12 ligand binding domains of *H. contortus* (3.1 ± 2.2 Å) and *Caenorhabditis elegans* (3.1 ± 2.2Å) both showed high structural similarity (TM-score: ≥ 0.97) to the published crystal structure for DAF-12 (ligand binding domain 3GYU_chainA; *Strongyloides stercoralis*) (Fig. 6.3B). Based on sequence and structural similarities, the biological processes for *Hc*-DAF-12 were predicted as DNA-templated regulation of transcription (GO:0006355), steroid hormone-mediated signalling pathway (GO:0043401) and ecdysone receptor-mediated signalling pathway (GO:0035076), with GO-scores of 0.94, 0.94 and 0.67, respectively.

6.4. Discussion

Using the enhanced genome and comprehensive transcriptome data sets for *H. contortus* as well as extensive information and data sets available for the related free-living nematode, *Caenorhabditis elegans*, we constructed a model for all four dauer signalling pathway components in *H. contortus* (Fig. 6.1). Based on this model, we explored gene transcription, expression and phosphorylation of the signalling molecules in all key developmental stages of *H. contortus*, compared the transcriptional profiles with those of stages in *Caenorhabditis elegans*, and examined the structural and likely functional similarities in a key determining point of the dauer signalling pathways, DAF-12, between these two nematodes. We believe that our model provides a basis to test hypotheses regarding the functions of these signalling pathway components in *H. contortus*, to elucidate the developmental biology of this and related nematodes, and to facilitate the discovery of new anthelmintic targets.

The identification of homologous GPCRs, ligands and receptors, kinases and phosphatases, transcriptional factors and a nuclear hormone receptor (DAF-12) in *H. contortus* implies relative conservation of dauer signalling, a statement which is, to some extent, supported by functional data for genes including *age-1*, *aap-1*, *daf-2*, *daf-7*, *daf-21*, *daf-16* and *pdk-1* in *H. contortus* (e.g., Gillan et al., 2009; Hu et al., 2010; McSorley et al., 2010; Li et al., 2014a, 2014b, 2016). Specifically, previous genetic complementation studies have shown that *Hc-daf-2*, *-daf-16* and *-daf-21* could at least partially rescue respective null mutants in *Caenorhabditis elegans* (see Gillan et al., 2009; Hu et al., 2010; Li et al., 2014b), indicating functionality in the regulation of developmental processes in *H. contortus*.

Although similarities in dauer signalling have been proposed (Tissenbaum et al., 2000; Beall and Pearce, 2002; Viney et al., 2005; Ogawa et al., 2009; Crook, 2014; Lok, 2016) and verified through in silico studies (Stoltzfus et al., 2012; Gilabert et al., 2016), there are some differences in the number of signalling homologues between *Caenorhabditis elegans* and parasitic nematodes. In the present study, although genes coding for pheromone receptors (*daf-37* and *daf-38*) and insulin-like peptides (i.e., *ins-1*, *ins-17* and *ins-18*) that promote dauer formation (Pierce et al., 2001; Ogawa et al., 2009; Zielinska

et al., 2009; McSorley et al., 2010; Borchert et al., 2012; Matsunaga et al., 2012a, 2012b; Murphy and Hu, 2013; Butcher, 2017) were identified in *H. contortus*, homologues coding for insulin-like peptides (e.g., *daf*-28, *ins-4* and *ins-6*) that promote larval development (Li et al., 2003; Murphy and Hu, 2013) were not detected. Such variation in the number of genes encoding signalling molecules (particular GPCRs, GTPase, insulin-like peptides, serine/threonine kinase and membrane permease) has been reported in previous studies of parasitic nematodes including *Ascaris suum*, *Nippostrongylus americanus* and *Strongyloides stercoralis* (see Stoltzfus et al., 2012; Gilabert et al., 2016). For example, *Strongyloides stercoralis* has only a small number of genes encoding insulin-like peptides, but has an expanded set of TGF-β-encoding genes (Stoltzfus et al., 2012). Although gene predictions depend significantly on the quality of the draft genomes used and bioinformatic algorithms applied (Korhonen et al., 2016; Laing et al., 2016), the differences seen here are likely explained by the divergent evolution of nematodes, reflecting the marked biological differences among nematodes, both free-living and parasitic (Crook, 2014; Gilabert et al., 2016; Viney and Harvey, 2017). Understanding the impact of evolutionary and biological divergences requires detailed studies of a broader range of nematodes species and functional assessments of particular dauer signalling components in them.

The dauer signalling pathways in *H. contortus* appear to play a role in larval diapause. The similar transcriptional patterns of signalling molecules between the infective L3 stage of *H. contortus* and the dauer stage of *Caenorhabditis elegans* indicate a similar function. The similarity in the arrested developmental state between the infective larva (L3) of strongylid nematodes and the dauer form in *Caenorhabditis elegans* has been discussed extensively (Hotez et al., 1993; Tissenbaum et al., 2000; Beall and Pearce, 2002), and the hypobiotic stage was proposed to be a pre-adaptation to parasitism (i.e., dauer hypothesis) (Crook, 2014), although we hasten to add that the L4 stage of some species of parasitic nematodes can undergo hypobiosis in host tissues and seems to be commensurate with dauer (Sommerville and Davey, 2002; Troell et al., 2006; Strube et al., 2007; Besier et al., 2016). This dauer hypothesis is supported by transcriptomic and proteomic evidence from the present study. Specifically, the transcriptional peaks of *daf-37* and *daf-38, daf-7, ins-1*, *ins-17* and *ins-18*, and *daf-16* in the L3 stage suggest a role in developmental arrest, whereas no translation and high transcription of genes *daf-9*, *daf-12* and *din-1*

imply an adaptation (e.g., pre-transcription and storage of RNA) for the subsequent parasitic stage of *H. contortus*. Interestingly, the transcription of *daf-7* peaks in L3s of *H. contortus* (clade V) and of parasitic nematodes representing other clades, including *Strongyloides stercoralis* (clade IV) and *Brugia malayi* (clade III); the transcription profiles in these nematodes is distinct from the free-living nematode *Caenorhabditis elegans* (clade V), suggesting a "repurposing" of roles from signalling to other biological processes, such as immunomodulation, in parasitic nematodes (Crook, 2014; Lok, 2016).

The roles of GPCRs, TGF-β, insulin-like ligands and fork head transcription factor (i.e., DAF-16) in dauer formation, and cytochrome P450 and nuclear hormone receptor (i.e., DAF-12) in regulating dauer formation in *Caenorhabditis elegans* have been reported previously (Pierce et al., 2001; Gerisch and Antebi, 2004; Matsunaga et al., 2012a, 2012b). However, there is a dearth of information on the functional roles of dauer signalling pathway components in developmental diapause of *H. contortus* (see Nikolaou and Gasser, 2006). Some explorations of parasitic nematodes, including *A. caninum* and *Strongyloides stercoralis* (see Hawdon and Datu, 2003; Datu et al., 2009; Stoltzfus et al., 2014; Albarqi et al., 2016), provide functional evidence for cGMP and IGF-1 signalling, and particularly for steroid hormone (DA) signalling, in regulating the activation of infective larvae. Other studies have suggested roles of signalling pathways in hypobiosis (at the L4 stage) in *H. contortus* (see Kooyman et al., 1995; Nikolaou and Gasser, 2006) and related nematodes including *Ostertagia ostertagi*, *Teladorsagia circumcincta* and *Dictyocaulus viviparus* (see Gibbs, 1986; Dopchiz et al., 2000; Strube et al., 2007; Strube et al., 2007; Baker et al., 2011; Laabs et al., 2012). Moreover, the different transcriptional profiles of signalling genes in L4 and/or adult stages between *H. contortus* and *Caenorhabditis elegans* might relate to variation in sexual differentiation between these species. Divergent transcription profiles of signalling molecules have also been reported for nematodes such as *Heterodera glycines*, *Heligmosomoides polygyrus* and *Strongyloides stercoralis* (see Elling et al., 2007; McSorley et al., 2010; Stoltzfus et al., 2012), suggesting biological divergence in larval diapause and reproductive development among nematode species. For example, two orphan genes *Hc-daf-22* and *Hc-fau* have been inferred to regulate larval diapause in *H. contortus* (see Yan et al., 2014; Guo et al., 2016). Clearly, functional analyses of signalling cascade components and their regulation

are required to improve our understanding of molecular pathways governing developmental processes in parasitic nematodes.

The structural and functional similarities of DAF-12 among free-living and parasitic nematodes suggest a common theme (i.e., a hormone-signalling module) in developmental regulation (Ogawa et al., 2009; Bento et al., 2010). Specifically, the central roles of steroids in the development of nematodes have been extensively reported and discussed, particularly the roles of DA-DAF-12 in regulating a connected network of genes (Motola et al., 2006; Hochbaum et al., 2011; Lee and Schroeder, 2012; Antebi, 2015; Wang et al., 2015; Butcher, 2017). Therefore, as a central point in the dauer signalling pathways, DAF-12 has been proposed as a therapeutic target in parasitic nematodes (Wang et al., 2009; Wang et al., 2017; Patton et al., 2018). In addition, non-coding small RNAs might play roles in regulating signalling pathways in *H. contortus* (see Chapter 5). For example, although substantial transcription of *Hc-daf-9* and *-daf-12* (i.e., genes involved in promoting reproductive development) (Antebi et al., 2000; Gerisch and Antebi, 2004) was detected in the L3 stage of *H. contortus*, the functions of these genes might be suppressed based on evidence of limited gene translation in this stage. In addition, the marked discrepancies in transcription and expression for genes such as *Hc-daf-21*, *-daf-7* and *-emb-8* might be explained by post-transcriptional regulation through microRNAs (miRNAs) (Filipowicz et al., 2008; Bartel, 2009), as tight regulation by such RNAs has been inferred recently for *H. contortus* (see Chapter 5). Nonetheless, our understanding of the biological roles of miRNAs in *H. contortus* is in its infancy (see Winter et al., 2012; Chapter 5), which contrasts the situation for *Caenorhabditis elegans* where the regulatory roles of miRNAs in development are well studied (Boehm and Slack, 2005; Ahmed et al., 2013). For instance, it has been reported that a complex feedback circuit between *daf-12* and the *let-7* family miRNAs regulates the division of epidermal stem cells during larval development (Bethke et al., 2009; Hammell et al., 2009).

In addition to post-transcriptional regulation, post-translational modifications are also likely involved in dauer signalling in *H. contortus*. This statement is supported by the present phosphoproteomic results. Phosphorylation was detected on *Hc*-DAF-21, -PPTR-1, -AKT-1, -DIN-1 and -GCK-2 in the L3 stage of *H. contortus*, indicating an activation of cGMP, insulin-like and steroid hormone signalling pathways. Specifically, the

exclusive phosphorylation of *Hc*-DAF-21, -DIN-1 and -GCK-2 in L3s suggests roles in arrested development. For *Caenorhabditis elegans*, it has been reported that phosphorylated PPTR-1 might activate the function of DAF-16 by reducing the inhibition of AKT-1, and that phosphorylated DIN-1 can inhibit the function of DAF-12 by forming a repression complex, promoting dauer diapause in this free-living nematode (Fielenbach and Antebi, 2007; Murphy and Hu, 2013). The identification of a range of kinases and their functional assessment in *H. contortus* also indicate extensive phosphorylation during signal transduction and integration and encourage future explorations of nematode tissues to better understand the roles of miRNAs, phosphorylation and lipids in signal transduction events and to discover novel anthelmintic targets. Clearly, available genetic technologies, 'omic resources, and informatic tools and hypotheses should facilitate such investigations in *H. contortus* and related parasitic worms.

In conclusion, we propose a model for the canonical (dauer-associated) cGMP, TGF-β, IGF-1 and steroid hormone signalling pathways from genomic and transcriptomic data sets for *H. contortus*, guided by resources and data available for *Caenorhabditis elegans*. This model should provide a basis to test hypotheses pertaining to these pathways and a platform for future functional explorations of molecular biological processes and associated regulatory mechanisms (via miRNAs, phosphorylations and/or lipids) involved in the development and reproduction of *H. contortus* and related nematodes as well as parasite-host cross-talk, which might guide the discovery of novel therapeutic targets. Although the focus of the present paper was on *H. contortus*, the approach employed for pathway reconstruction could be applied to other nematodes that are relatively closely related to *Caenorhabditis elegans*, including, for instance, *Ostertagia* and *Trichostrongylus* (clade V), and *Ascaris* and *Toxocara* (clade III).

6.5. References

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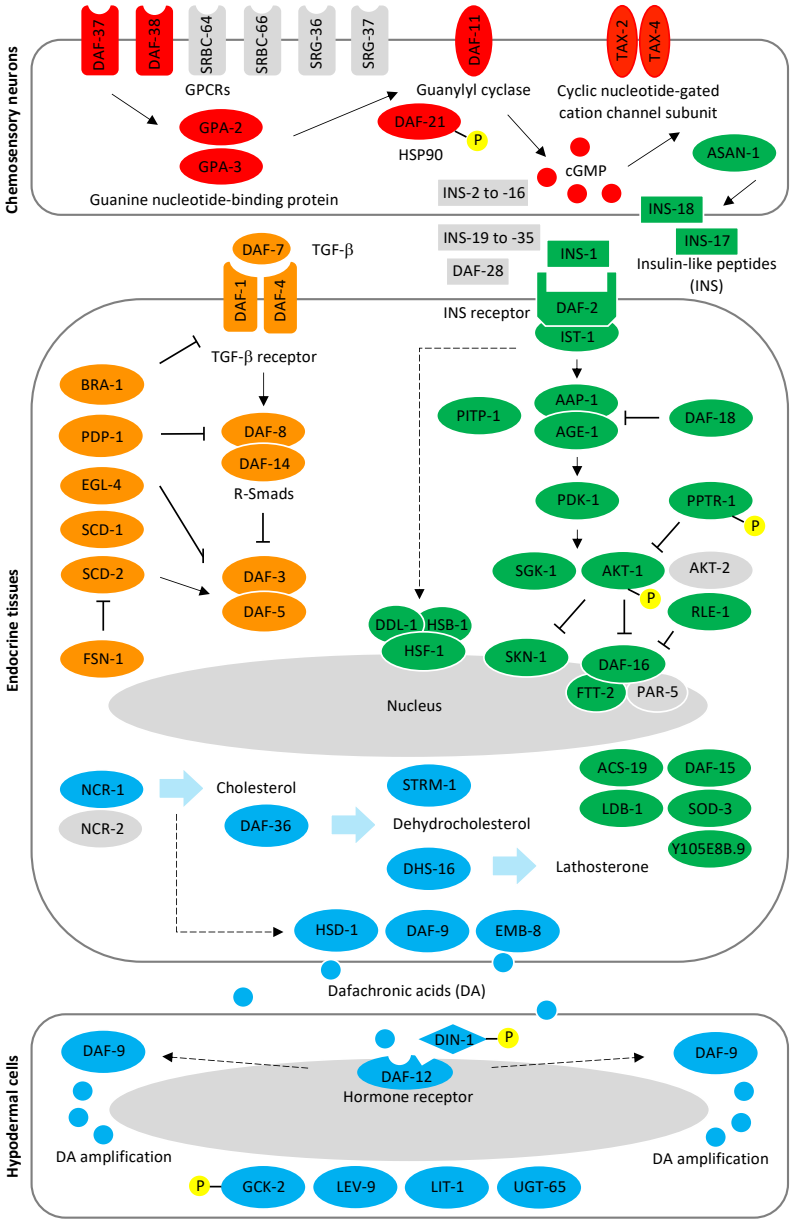


Fig. 6.1. Dauer signalling pathways model proposed for *Haemonchus contortus*. The cGMP (red), TGF-β (orange), insulin-like growth factor 1 (IGF-1) (green) and steroid hormone (light blue) signalling pathways are constructed based on information and data available for *Caenorhabditis elegans*. Pathway components not identified in *H. contortus* are indicated in grey. A likely scenario is that environmental signals are received by G protein-coupled receptors (GPCRs) through cGMP signalling in chemosensory neurons, transduced via TGF-β and insulin-like peptides in paralleled TGF-β signalling and IGF-1 signalling, which then converge into the steroid hormone signalling pathway via the biosynthesis of dafachronic acids (DAs; ligands of nuclear hormone receptor DAF-12) in neuroendocrine tissues, determining the activation of DAF-12 in target (hypodermal) cells. The activation of DAF-12 initiates or regulates numerous molecular events such as transcription. Signalling or regulation is indicated by an arrow or a line with bar. Phosphorylation events detected in the third larval stage (L3) of *H. contortus* are indicated by phosphate group (yellow). Dashed arrows indicate pathways which need to be further elucidated.

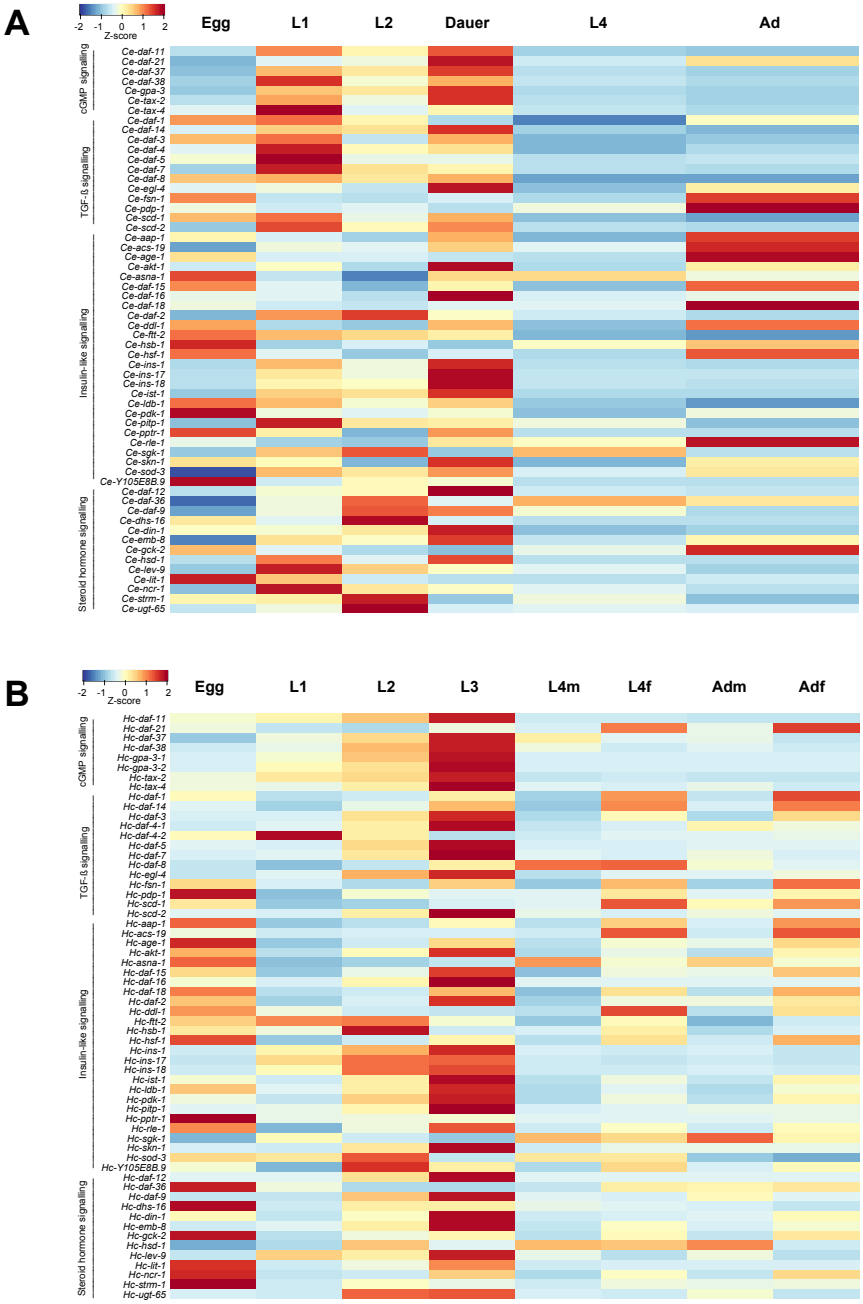


Fig. 6.2. Transcription of dauer signalling genes in *Caenorhabditis elegans* and in *Haemonchus contortus*. Messenger RNA transcriptional levels (fragments per kilobase per million mapped reads, FPKM) of (A) 60 dauer signalling genes in key developmental stages of *Caenorhabditis elegans* [egg, the first (L1), second (L2), dauer, fourth (L4) – stage larvae and adults (Ad)], and (B) all 61 homologous genes in *H. contortus* stages [egg, L1, L2, the third (L3), L4 (female and male L4s - L4f and L4m), and Ad (female and male adults - Adf and Adm)] are indicated in the heat maps. Colour scales and Z-scores indicate scaled FPKMs in the rows. Genes involved in the cGMP, TGF-β, IGF-1 and steroid hormone signalling pathways are listed.

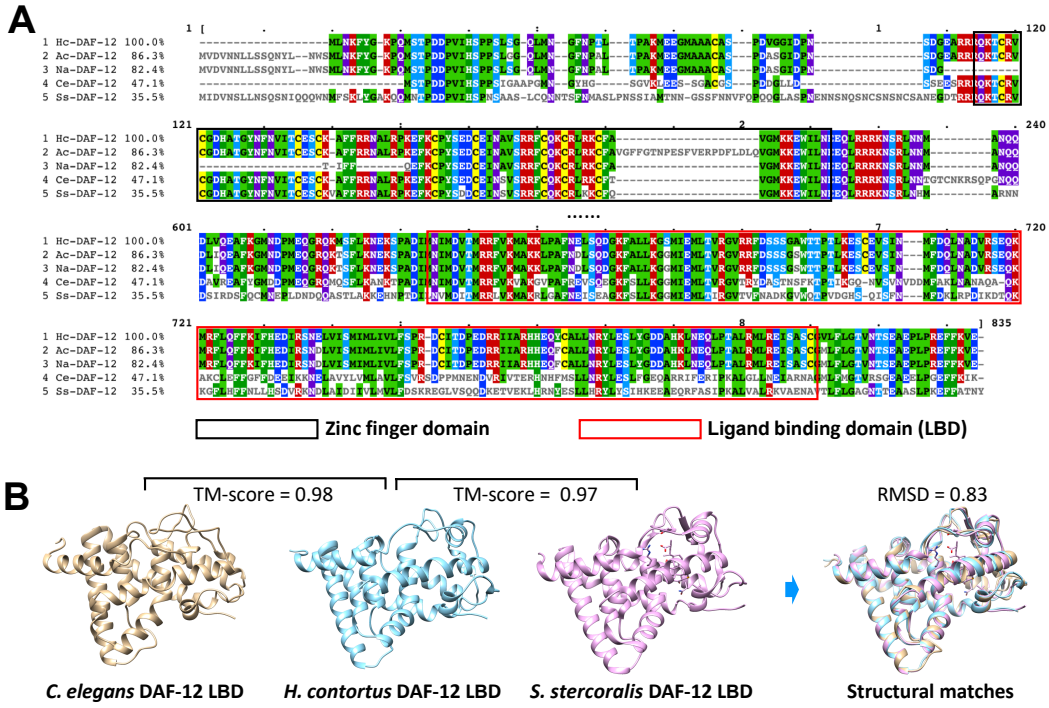


Fig. 6.3. DAF-12 sequence and structural similarities among nematodes. (A) Alignment of the inferred protein sequences of DAF-12 of *Haemonchus contortus* (*Hc*-DAF-12), *Ancylostoma ceylanicum* (accession no. EPB79655), *Necator americanus* (accession no. XP_013307217), *Caenorhabditis elegans* (WormBase ID. CE27585) and *Strongyloides stercoralis* (GenBank accession no. AAD37372) is shown, with sequence similarities indicated. Zinc finger domain and ligand binding domain (LBD) are framed in black and red, respectively. (B) Structure models of LBDs of *H. contortus* and *Caenorhabditis elegans* are matched to the published crystal structure (PDB accession no. 3GYU_ChainA; *Strongyloides stercoralis*); TM-scores (0.98 and 0.97) and an overall root-mean-square deviation (RMSD) of 0.83 indicate high pairwise structural similarities.

Supplementary Table S6.1. Salient information and data for genes known or inferred to be involved in the dauer signalling pathway in *Caenorhabditis elegans*.

Supplementary Table S6.2. Dauer signalling gene homologues in *Haemonchus contortus*.

Supplementary Table S6.3. Gene transcription of dauer signalling gene homologues in key developmental stages of *Haemonchus contortus*.

Supplementary Table S6.4. Protein expression and phosphorylation of dauer signalling gene homologues in key developmental stages of *Haemonchus contortus*.

Chapter 7. Dafachronic acid promotes larval development in *Haemonchus contortus* by modulating dauer signalling and lipid metabolism

Abstract

Here, we discovered an endogenous dafachronic acid (DA) in the socioeconomically important parasitic nematode *Haemonchus contortus*. We demonstrate that DA promotes larval exsheathment and development in this nematode via a relatively conserved nuclear hormone receptor (DAF-12). This stimulatory effect is dose- and time-dependent, and relates to a modulation of dauer-like signalling, and glycerolipid and glycerophospholipid metabolism, likely via a negative feedback loop. Specific chemical inhibition of DAF-9 (cytochrome P450) was shown to significantly reduce the amount of endogenous DA in *H. contortus*; compromise both larval exsheathment and development in vitro; and modulate lipid metabolism. Taken together, this evidence shows that DA plays a key functional role in the developmental transition from the free-living to the parasitic stage of *H. contortus* by modulating the dauer-like signalling pathway and lipid metabolism. Understanding the intricacies of the DA-DAF-12 system and associated networks in *H. contortus* and related parasitic nematodes could pave the way to new, nematode-specific treatments.

7.1. Introduction

Dafachronic acids (DAs) are bile acid-like, steroidal hormones, which were first discovered in the free-living nematode *Caenorhabditis elegans* (see Motola et al., 2006). In this nematode, Δ7-DA has been shown bind to the nuclear receptor DAF-12 in *Caenorhabditis elegans* to modulate developmental and reproductive processes in response to changing environmental conditions (Gerisch et al., 2004; Gerisch et al., 2007; Bethke et al., 2009; Hammell et al., 2009; Schaedel et al., 2012; Mahanti et al., 2014; Wang et al., 2015). For instance, under favourable conditions, the DA-DAF-12 module is activated to promote continuous larval development to the adult stage, whereas under unfavourable conditions, this module is inactivated to suppress development, leading to larval arrest (called dauer formation or diapause) (Hu, 2007; Fielenbach and Antebi, 2008; Schaedel et al., 2012). This DA-DAF-12 system - essentially a "developmental switch" - is regulated by the dauer signalling pathway, which comprises elements of the cyclic guanosine monophosphate (cGMP), DAF-7 transforming growth factor-β (TGF-β), DAF-2 insulin/insulin-like growth factor 1 (IGF-1) and steroid-hormone signalling cascades (Hu, 2007; Fielenbach and Antebi, 2008; Lee and Schroeder, 2012).

The DA-DAF-12 system is not unique to *Caenorhabditis elegans*. Some studies have shown that this endocrine system occurs and is functional in *Pristionchus pacificus* - another free-living nematode (see Ogawa et al., 2009), and in selected parasitic nematodes such as *Ancylostoma* and *Strongyloides* spp. (see Wang et al., 2009; Stoltzfus et al., 2012, 2014; Zhi et al., 2012; Wang et al., 2015; Albarqi et al., 2016). Other studies have revealed components of the DA-DAF-12 system in various other parasitic nematodes representing different evolutionary clades, including *Trichinella spiralis*, *Trichuris trichiura* (clade I), *Brugia malayi* and *Loa loa* (clade III), and *Haemonchus contortus* (clade V) (see Chapter 6) using informatic approaches (Gilabert et al., 2016). Collectively, this published information indicates that this endocrine system (controlling dauer formation, or developmental arrest) is relatively conserved in, and specific to, members of the phylum Nematoda (Viney, 2009; Crook, 2014), raising interest in the proposition that DAF-12 and/or associated molecules might represent suitable targets for new anthelmintics (cf. Wang et al., 2009, 2017; Patton et al., 2018). This aspect is particularly important, given the nature and extent of anthelmintic resistance in

socioeconomically important parasitic nematodes of animals, and the adverse impact that it has on the agricultural and associated industries through reduced animal productivity (Kotze and Prichard, 2016). However, surprisingly, as yet there has been no detailed structural or functional investigation of DA-DAF-12 and associated signalling pathways in economically significant nematodes of livestock animals.

The barber's pole worm, *H. contortus* (order Strongylida), is particularly well-suited for molecular explorations (Gasser et al., 2016). It is arguably the most pathogenic nematode of ruminants, and the disease that it causes (haemonchosis) has a major, adverse impact on animal health and production worldwide (Roeber et al., 2013; Besier et al., 2016). This worm has a short life-cycle (~28-30 days), has major reproductive potential and, thus, can be readily produced in large numbers in experimental sheep, allowing detailed in vitro studies. The worm develops from the egg to the adult stage through four larval stages (Veglia, 1915), with a dauer-like developmental arrest at the third stage (L3) in the environment, and a possible developmental arrest (hypobiosis) at the fourth stage (L4) within the host animal (Gibbs, 1986; Besier et al., 2016; Nisbet et al., 2016). Recently, we established an efficient in vitro-culture system for larval stages of this parasitic nematode (see Preston et al., 2015), which facilitates in-depth studies of developmental processes and mechanisms (see Chapters 2-6), underpinned by extensive genomic resources (Laing et al., 2013; Schwarz et al., 2013; Doyle et al., 2018) and enabled by a ready accessibility to transcriptomic, proteomic, lipidomic and informatic technologies (e.g., Korhonen et al., 2016; see Chapters 2-4). Using these resources and technologies, in the present study, we elucidate the functionality of DA-DAF-12 system and explore how it modulates associated pathways in this highly significant parasitic nematode - *H. contortus*.

7.2. Materials and methods

7.2.1. Ethics approval

Haemonchus contortus (Haecon-5 strain) was produced in Merino lambs (6 months of age; Victoria, Australia), maintained under helminth-free conditions in facilities in the University of Melbourne. The procedures for animal maintenance and experiments were

approved by the University of Melbourne (permit no. 1714374), which follows Part 3 of the Prevention of Cruelty to Animals Act 1986 and Part 4 of the Prevention of Cruelty to Animals Regulations 2008 of the State of Victoria as well as the Australian Code for the Care and Use of Animals for Scientific Purposes (1969).

7.2.2. H. contortus stages

A monospecific infection of *H. contortus* was maintained in sheep under well-controlled experimental conditions (Nikolaou et al., 2002); three distinct larval stages of this nematode were produced in vitro using established methods (Preston et al., 2015). In brief, third-stage larvae (L3s) were collected from coproculture, purified and maintained at 10 °C in a refrigerated incubator; exsheathed L3s (xL3s) were produced using a well-established hypochlorite-treatment method (Preston et al., 2015); and xL3s were cultured (300 per well of 96-well culture plates), under standardised conditions, in Luria Bertani medium (LB) supplemented with Antibiotic-Antimycotic (cat no. 15240-062, Gibco) (LB*) at 38 °C, 10% v/v CO_2 to yield fourth-stage larvae (L4s) of *H. contortus*.

7.2.3. Detection of DA in the worm

Endogenous DA was identified by liquid chromatography-mass spectrometric (LC-MS) analysis of lipids extracted from three distinct developmental stages of *H. contortus*. Lipids were extracted from four replicates (each 1 mg dry weight) of each L3s, xL3s and L4s using an established method (see Chapter 4). Each replicate was suspended in ice-cold methanol (40%), homogenised using zirconium oxide beads (ZROB05, Next Advance, USA) and extracted with chloroform:methanol (2:1) by centrifugation at 10,000 ×g for 15 min, dried and resuspended in methanol (100%), then subjected to LC-MS analysis in an Orbitrap Fusion Lumos mass spectrometer coupled to an Ultimate 3000 UHPLC (Thermo Fisher Scientific, San Jose, CA, USA). Endogenous DA were identified by comparison with the reference standards (25S)-Δ7-DA (see Sharma et al., 2009) and (25S)-Δ4-DA (cat. no. 23017-97-2; Cayman Chemical, USA) (exact mass: 413.3061).

7.2.4. Structure modelling and DAF-12 reporter assay

The structure of the ligand-binding domain (LBD) of DAF-12 of *H. contortus* (*Hc*-DAF-12) (using the inferred amino acid sequence: GenBank accession no. MK_256962) was modelled using the program I-TASSER (Yang et al., 2015) and compared with that of *A. caninum* (*Ac*-DAF-12) (Wang et al., 2009) using UCSF Chimera v.1.12 (Pettersen et al., 2004). Structural similarities between query and template sequences were established using sequence length, overall root-mean-square deviation (RMSD) and structural similarity scores (SDM and Q-score). To test whether *Hc*-DAF-12 can be activated by (25S)-Δ7-DA, a well-established reporter assay was performed as described previously (Motola et al., 2006; Wang et al., 2009). In brief, HEK293 cells were transfected with the luciferase reporter (50 ng), CMX-β-galactosidase reporter (10 ng) and *Hc*-DAF-12 expression plasmids (15 ng). Ethanol or (25S)-Δ7-DA (0 to 1 μM) was added to cells (8 h following transfection) and incubated for 16 h. Luciferase activity was measured with reference to a CMX-β-galactosidase control.

7.2.5. Assaying the effect of (25S)-Δ7-DA on larval exsheathment and development

First, L3s (300 worms per well; four replicates) were exsheathed by incubating them at 38 °C and 10% v/v CO_2 for 48 h in physiological saline in the presence or absence of 10 μM of (25S)-Δ7-DA. The number and percentage of exsheathed L3s (xL3s) were assessed every 12 h. Second, xL3s (300 worms per well; four replicates) were cultured to L4s at 38 °C, 10% v/v CO_2 in LB* in the absence or presence of (25S)-Δ7-DA (10 μM to $10*2^{-17}$ μM). The numbers of xL3s and L4s in culture were calculated every 24 h, and the proportion of L4s calculated at each time point. Statistical analyses (student's t-test, Spearman's rank correlation and non-linear regression) were performed using Prism 7 (GraphPad, La Jolla, USA).

7.2.6. Transcriptomic, proteomic and lipidomic analyses

The transcriptomes, proteomes and lipidomes were produced from *H. contortus* xL3s, which had been exsheathed using the established hypochlorite-treatment method (Preston

et al., 2015) and then incubated in LB* (38 °C, 10% v/v CO_2 for 24 h) in the presence or absence of 1.25 μM (25S)-Δ7-DA were produced and compared. For each treatment, four replicates of 30,000 xL3s each were incubated at 38 °C, 10% v/v CO_2 for 24 h.

For transcriptomic analysis, total RNA was extracted from each of the replicates of xL3s, processed and sequenced as described previously (see Chapter 5). In brief, strand-specific mRNA libraries were constructed using the TruSeq RNA Library Prep Kit (Illumina) and sequenced on the BGISEQ-500 platform. Raw reads were processed and mapped to predicted genes of *H. contortus* (BioProject: PRJEB506) using Bowtie v.2.1.0 (Langmead and Salzberg, 2012) within the software package RSEM v.1.2.11 (Li and Dewey, 2011).

For proteomic analysis, proteins were isolated from the replicates as described previously (see Chapters 2 and 5). In brief, protein (50 μg) samples were reduced with Tris(2-carboxyethyl)phosphine (TCEP), alkylated with iodoacetamide and digested with Lys-C/trypsin Mix (cat no. V5072; Promega, USA). The digested samples were acidified with 1.0% (v/v) formic acid and purified using Oasis HLB cartridges (cat no. 186000383; Waters, USA) and subjected to LC-MS/MS analysis using a Q Extractive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000). Mass spectrometry data were analysed using MaxQuant to identify and quantify peptides (Tyanova et al., 2016a).

For lipidomic analysis, lipids were extracted from the replicates and analysed by LC-MS/MS using an Orbitrap Fusion Lumos mass spectrometer (see Chapter 4). For the semi-quantitation of identified lipids, the Splash® Lipidomix® Mass Spec. Standard (cat no. 330707-1EA, Avanti Polar Lipids, USA), including phosphatidylcholine [PC, 15:0_18:1(d7)], phosphatidylethanolamine [PE, 15:0_18:1(d7)], phosphatidylserine [PS, 15:0_18:1(d7)], phosphatidylglycerol [PG, 15:0_18:1(d7)], phosphatidylinositol [PI, 15:0_18:1(d7)], lysophosphatidylcholine [LPC, 18:1(d7)], lysophosphatidylethanolamine [LPE, 18:1(d7)], monoradylglycerol [MG, 18:1(d7)], diradylglycerol [DG, 15:0_18:1(d7)] and triacylglycerol [TG, 15:0_18:1(d7)_15:0], was used as the internal standard. Additional PS[15:0_18:1(d7)], MG[18:1(d7)] and DG[15:0_18:1(d7)] were supplemented to reach a final concentration of 100 μg/ml for each lipid species. Lipids

were identified and quantified using LipidSearch software v.4.2.20 (Thermo Scientific), and manually curated.

The mRNAs, proteins and lipids quantified were subjected to principal component and hierarchical cluster analyses using the Perseus computational platform (Tyanova et al., 2016b; Tyanova and Cox, 2018). Differential transcription was explored using the limma, glimma and edgeR packages (Law et al., 2016); a fold-change (FC) of ≥ 2 and a false discovery rate (FDR) of ≤ 0.01 defined a significant difference, unless otherwise stated (FC ≥ 2 and $P \leq 0.01$). Differential protein expression analysis was conducted using the program Perseus v.1.6.1.1, employing an FC of ≥ 1.5 and an FDR ≤ 0.05 as thresholds. For lipids, an FC of ≥ 1.5 and a P value of ≤ 0.01 were used as cut-offs. Differentially transcribed mRNAs and expressed proteins were assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) terms using BlastKOALA (Kanehisa et al., 2016), and KEGG annotations were analysed and displayed using FuncTree 2 (Uchiyama et al., 2015).

7.2.7. Inhibition of DAF-9 (cytochrome P450) in the worm

Dafadine A (cat. no. SML0736; Sigma-Aldrich) is known to specifically inhibit DAF-9 in *Caenorhabditis elegans* (see Luciani et al., 2011). This chemical was used to inhibit the endogenous production of DAs. In brief, worms (300 L3s or xL3s per well) were exposed to dafadine A (100 μM) in LB* and incubated at 38 °C, 10% CO_2 for 7 days. DMSO, (25S)-Δ7-DA (1.25 μM), and dafadine A (100 μM) + (25S)-Δ7-DA (1.25 μM) were used as different controls. DA levels, larval development and lipid abundances were analysed between treated and untreated worms.

7.3. Results

7.3.1. Transcriptional changes linked to the dauer-signalling pathway during larval transition, and the identification and quantitation of endogenous Δ7-DA

Haemonchus contortus undergoes a morphological transition from an infective L3, via the exsheathed L3 (called xL3), to the parasitic L4 stage (Veglia, 1915), which can be

carried out in vitro (Preston et al., 2015). Here, we investigated, the transcription of genes inferred to be linked to dauer-signalling during this transition in vitro, and then identified and quantitated Δ7-DA in respective larval stages of the nematode.

We recorded significant alterations in the transcription of 14 of 61 genes inferred to be involved in dauer signalling in *H. contortus* (Fig. 7.1A and 1B): three genes (*Hc-daf-21* [or *hsp-90*], *-scd-1* and *-hsb-1*) were highly upregulated in xL3s *versus* L3s, one (*Hc-daf-36*) was highly upregulated in L4s *versus* xL3s, and 10 gene (*Hc-gpa-2*, *-gpa-3*, *-daf-7*, *-daf-3*, *-scd-2*, *-akt-1*, *-daf-16*, *-emb-8*, *-daf-12* and *-ugt-65*) were substantially downregulated in L4 versus xL3s (FC > 2, $P < 0.01$; Supplementary Table S7.1).

Based on prior knowledge for *Caenorhabditis elegans* (see Hu, 2007), these transcriptional alterations (Fig. 7.1B) suggested that DA(s) are integral to this developmental transition in *H. contortus*, because the biosynthesis of DAs likely represents the outcome of the dauer signalling pathway (see Chapter 6). Therefore, we investigated *H. contortus* L3s for the presence of DA. Endogenous Δ7-DA (retention time: 4.2 min; mass error: ~0.5 part per million) was unequivocally identified in L3s (Fig. 7.1C), and then quantified in all larval stages studied here (Fig. 7.1D). The abundance of Δ7-DA increased substantially from L3 to xL3 (24 h following exsheathment) and then decreased gradually in the ensuing 6 days of in vitro-culture (Fig. 7.1D).

7.3.2. Synthetic, exogenous (25S)-Δ7-DA activates Hc-DAF-12

To examine whether (25S)-Δ7-DA might bind LBD of *Hc*-DAF-12, we compared the structural model of this predicted LBD with that of *Ac*-DAF-12 from *Ancylostoma caninum* (a canine hookworm which is a related strongylid nematode) (cf. [13]). Using three independent algorithms, we showed high structural similarity, achieving a root-mean-square deviation (RMSD) of 1.05, a structural distance measure (SDM) of 20.89 and a Q-score of 0.88 (Fig 2A), suggesting that *Hc*-DAF-12 and *Ac*-DAF-12 have a similar binding affinity and ability to activate DAF-12. This proposal was confirmed by showing that, in a luciferase reporter assay, (25S)-Δ7-DA at 50 nM to 1 μM activated *Hc*-DAF-12 with an EC_{50} of 12.54 nM, which is similar to that of *Ac*-DAF-12 (12.80 nM) (Fig 2B).

7.3.3. Exogenous (25S)-Δ7-DA stimulates larval growth and development

Knowing that (25S)-Δ7-DA activates *Hc*-DAF-12, we then explored whether we could influence larval exsheathment, growth and/or development in vitro using exogenous, synthetic (25S)-Δ7-DA. There was no significant difference ($P > 0.05$) in exsheathment between L3s and L3s exposed for 48 h to 10 μM of (25S)-Δ7-DA (Fig. 7.2C). However, when cultured for 48 h in the presence of 10 μM of (25S)-Δ7-DA, 49% more xL3s developed to L4s in vitro, and the development from xL3s to L4s was significantly more rapid than unexposed controls (7 days) (Fig. 7.2D). This stimulatory effect of (25S)-Δ7-DA on larval growth and development was both dose- and time-dependent (Fig. 7.2E and 7.2F). There was a positive correlation between the concentration ($r = 0.95$, $P < 0.001$) of (25S)-Δ7-DA (0-1.25 μM) and larval development (82-96%) after 2 days of culture, and (25S)-Δ7-DA of 1.25, 2.50, 5.00 and 10.00 μM achieved a similar development rate (Fig. 7.2E). Under the condition of treatment with 1.25 μM of (25S)-Δ7-DA, there was a positive correlation ($r = 0.99$, $P < 0.05$) between the time of treatment (0-48 h) and larval development (83-96%) (Fig. 7.2F). The half maximum effective concentration (EC_{50}) of (25S)-Δ7-DA on larval development was estimated at 320 nM.

7.3.4. Establishment of transcriptomic, proteomic and lipidomic data sets for subsequent analyses

To explore molecular responses in *H. contortus* associated with DA, we established transcriptomic, proteomic and lipidomic resources. Individual transcriptomic, proteomic and lipidomic data sets were produced for xL3s (0 h), xL3s (24 h) and (25S)-Δ7-DA-treated xL3s (24 h) (four replicates each) (Fig. 7.3; Supplementary Fig. S7.1A). The 'larval' transcriptome, proteome and lipidome comprised 12,217 mRNAs, 1425 protein groups and 653 lipids (representing 23 classes), respectively; proteins were detected for 10% of all transcripts identified. Principal component analyses showed that the three data sets clustered into three distinct groups [xL3s (0 h), xL3s (24 h) and (25S)-Δ7-DA-treated xL3s (24 h)], and hierarchical cluster analyses indicated differences in mRNA transcription, protein expression and lipid abundance between or among the groups (Fig. 7.3; Supplementary Fig. S7.1B and S1C).

7.3.5. Analysis of differential transcription, protein expression and lipid abundance

Using individual transcriptomic, proteomic and lipidomic data sets produced (Supplementary Tables S7.2-7.4), we studied molecular changes in *H. contortus* xL3s and xL3s exposed to (25S)-Δ7-DA for 24 h. Extensive changes in mRNA transcription, protein expression and lipid abundance were recorded (Supplementary Tables S7.2-7.4). Specifically, significantly higher levels of 1055 mRNAs, 101 proteins and 180 lipids, and significantly lower levels of 1029 mRNAs, 46 proteins and 109 lipids were detected in xL3s (at 24 h) compared with L3s immediately following exsheathment (Fig. 7.3A-C; Supplementary Tables S7.2-7.4). More differences were seen in xL3s exposed to (25S)-Δ7-DA at 24 h, including significantly increased levels of some mRNAs (n = 1378), proteins (n = 263) and lipids (n = 177) and significantly decreased levels of other mRNAs (n = 1362), proteins (n = 126) and lipids (n = 109) (Supplementary Tables S7.2-7.4). Most significant molecular changes detected in xL3s (at 24 h) were identified in (25S)-Δ7-DA-treated xL3s (at 24 h); these changes were inferred to be associated with biological processes including environmental information processing (principally signal transduction), genetic information processing (including folding, sorting and degradation) and lipid metabolism (including fatty acid degradation and steroid hormone biosynthesis) (Fig. 7.3D; Supplementary Fig. S7.2). Changes in lipid metabolism related predominantly to sphingolipids (ceramide and sphingomyelin), glycerolipids (DG and TG) and glycerophospholipids (PA, PC, PE, PG, PI and PS) (Fig. 7.3D; Supplementary Table S7.4).

7.3.6. (25S)-Δ7-DA modulates transcription in genes associated with dauer signalling genes and larval growth

First, we explored differential transcription for genes associated with dauer signalling (Fig. 7.4). In the transition from L3 to xL3s, 24 h following L3 exsheathment, when endogenous DA is at its highest level in xL3s (Fig. 7.1D), transcription in the dauer signalling cascade was downregulated (FC ≥ 2 and P < 0.01) for 14 genes involved in cGMP (*Hc-gpa-2* and *-gpa-3*), TGF-β (*Hc-daf-7*, *-daf-4*, *-daf-5* and *-scd-2*), IGF-1

signalling (*Hc-ins-1*, *-ins-18*, *-ist-1*, *-daf-16*, *-skn-1* and *-acs-19*) or steroid hormone signalling (*Hc-emb-8* and *-daf-12*), and transcription was upregulated (FC ≥ 2 and $P < 0.01$) one gene (*Hc-scd-1*) associated with TGF-β signalling and four genes (*Hc-daf-36*, *-daf-9*, *-hsd-1* and *-lev-9*) linked to steroid hormone signalling (Fig. 7.4; Supplementary Table S7.5). In order to assess whether DA biosynthesis alters the differential transcription of these genes, we undertook an experiment where we added exogenous (25S)-Δ7-DA (1.25 μM) to xL3s for 24 h (after exsheathment). The results showed a further, significant reduction (FC ≥ 2 and $P < 0.01$) in the transcription of 12 downregulated genes (*Hc-gpa-2*, *-gpa-3*, *-daf-7*, *-daf-4*, *-daf-5*, *-scd-2*, *-ins-1*, *-ins-18*, *-ist-1*, *-daf-16*, *-skn-1* and *-daf-12*) and two upregulated genes (*Hc-scd-1* and *-daf-36*) (Fig. 7.4; Supplementary Table S7.5).

Beyond the dauer signalling pathway, (25S)-Δ7-DA supplementation induced significant ($P \leq 0.05$) molecular alterations in xL3s following exsheathment. Although a downregulation was recorded exclusively for mRNA transcription of genes *cat-4*, *cox-6A*, *hil-1*, *hsp-16.1*, *lgc-34* and *ttr-17*, an upregulation was measured for mRNA transcription (genes *clec-48*, *cyp-14A5*, *osta-3* and *pgp-1*), protein expression (SYM-1 and LPR-3) and lipid abundance [for DG(15:0_18:1), TG(15:0_10:0_18:2), PC(15:0_20:4), PC(16:0_17:0), LPC(15:0) and PI(15:0_20:4)] (Fig. 7.5A-C; Supplementary Tables S7.2-7.4). These differentially transcribed mRNAs and upregulated proteins were inferred to be involved in larval development, pharynx development and the attachment of body muscle to the extracellular cuticle (body morphogenesis) (Fig. 7.5D; Supplementary Tables S7.2 and S7.3). The altered abundances of particular glycerolipids and glycerophospholipids were associated with cellular proliferation, lipid signalling and metabolism (Fig. 7.5D).

7.3.7. Inhibition of endogenous Δ7-DA by dafadine A compromises larval exsheathment and development, and alters lipid abundance

As a previous study (Luciani et al., 2011) has shown that dafadine A can specifically inhibit the biosynthesis of DAs in *Caenorhabditis elegans*, we elected to test the effect of this inhibitor on Δ7-DA biosynthesis, larval exsheathment and development of *H. contortus*. Treatment with dafadine A (100 μM) slowed larval development from the L3

to the L4 stage and significantly reduced the level of endogenous Δ7-DA in dafadine A-treated worms, compared with untreated and (25S)-Δ7-DA-treated worms (Fig. 7.6A-B). When L3s were exposed to 100 μM of dafadine A in vitro, exsheathment was significantly inhibited ($P < 0.001$) compared with unexposed L3 controls (Fig. 7.6C). Similarly, when xL3s were exposed to 100 μM of dafadine A, larval development decreased significantly ($P < 0.001$) (Fig. 7.6D). The inhibitory effects of dafadine A on the production of endogenous Δ7-DA, larval exsheathment and development were partially or completely "rescued" by supplementation with 1.25 μM of (25S)-Δ7-DA ($P < 0.001$) (Fig. 7.6B-D).

Since the altered abundances of particular glycerolipids and glycerophospholipids were linked to (25S)-Δ7-DA treatment (Fig. 7.5D), we explored the relationship between these molecules and DA in *H. contortus*. Treatment with dafadine A (100 μM) altered the abundances of DG(15:0_18:1), TG(15:0_10:0_18:2), PC(15:0_20:4; 16:0_17:0), LPC(15:0) and PI(15:0_20:4) in xL3s at 24 h. Specifically, dafadine A significantly ($P < 0.01$) increased the levels of DG(15:0_18:1) and TG(15:0_10:0_18:2); the levels of these lipids increased further when exposed to (25S)-Δ7-DA ($P < 0.05$) with reference to untreated controls (Fig. 7.7A and 7.7B). It was also evident that treatment with dafadine A for 24 h significantly reduced levels of PC (15:0_20:4, 16:0_17:0), LPC (15:0) and PI (15:0_20:4) ($P < 0.05$) compared with untreated controls, which were reversed by supplementation with 1.25 μM of (25S)-Δ7-DA (Fig. 7.7C-F).

7.4. Discussion

This study identified, for the first time, DA in the strongylid nematode *H. contortus*, and showed that this hormone promotes larval exsheathment and development via a relatively conserved nuclear hormone receptor, DAF-12. In *H. contortus*, the DA-DAF-12 complex modulates the dauer-like signalling pathway, via a negative feedback circuit, and effects molecular alterations linked to pharynx development, body morphogenesis, cellular growth, lipid signalling and metabolism.

The Δ7-DA signal that induces larval development in *H. contortus* is transduced via DAF-12. Since the relationship between DA-DAF-12 and developmental regulation in *Caenorhabditis elegans* is well-established (Beckstead and Thummel, 2006; Bethke et

al., 2009; Hammell et al., 2009), a conserved DA-DAF-12 module had been proposed for parasitic nematodes (Wang et al., 2009; Bento et al., 2010). Recently, we also showed quite marked sequence and/or structural similarity in the inferred DAF-12 ligand-binding domain (LBD) between *H. contortus* and other individual strongylids (*A. ceylanicum* and *Necator americanus*) or rhabditids (*S. stercoralis* and *C. elegans*) (see Chapter 6), suggesting relative functional conservation in dafachronic acid binding and signalling. Indeed, here we confirmed that (25S)-Δ7-DA activates *Hc*-DAF-12 in a luciferase reporter assay, with an EC_{50} (12.54 nM) that is similar to for *Ac*-DAF-12 (12.80 nM) from *A. caninum*. These findings suggest that the endogenous Δ7-DA signal is transduced by DAF-12 to promote larval development in *H. contortus*, consistent with *Ancylostoma* and *Strongyloides* spp. (cf. Ogawa et al., 2009; Wang et al., 2009; Zhi et al., 2012; Albarqi et al., 2016).

It has been reported that the binding of DA to DAF-12 in parasitic nematodes is similar to that of bile acids to the farnesoid X receptor in mammals, suggesting that a bile acid-like signalling pathway exists in parasitic nematodes (Zhi et al., 2012). Interestingly, a common hormone-theme has been proposed for physicochemical communications between parasite and host animal (Beckage, 1991; Lok, 2016; Butcher, 2017). A good example of this is that prolactin evokes the transmammary transmission of larvae of the ascaridoid nematode *Toxocara canis* in mice (Jin et al., 2008). It is readily possible that the DA-DAF-12 module in the latter nematode plays a role in regulating or signalling larval activation and transmission, but this involvement needs to be verified molecularly. Clearly, understanding the functionality of the DA-DAF-12 module in parasitic nematodes could provide a paradigm for exploring cross-talk between parasite and host, particularly for worms which can enter into or exit from hypobiosis (arrested development) in their host, such as some members of the families Trichostrongylidae and Ascarididae (Michel, 1974; Gibbs, 1986).

In *H. contortus*, the upregulated transcription of particular dauer signalling genes during the developmental transition from the L3 (dauer-like) stage to the L4 stage indicates an active DA biosynthesis in xL3s, confirmed by measuring an increase in the level of Δ7-DA following L3 exsheathment. These alterations are similar to the transcriptional changes and hormone signal amplification seen in *C. elegans* during its development to the reproductively-active adult stage (see Lee and Schroeder, 2012;

Schaedel et al., 2012). By contrast, a decreased level of Δ7-DA during the ensuing larval development indicates a reduction of its biosynthesis, which is supported by the observation of a pronounced downregulation of transcription of particular genes linked to dauer-like signalling. The dynamics of these changes in DA and transcription levels suggest that the endogenous synthesis of Δ7-DA is relatively tightly modulated or controlled via an, as yet, uncharacterised feedback circuit. A similar negative feedback mechanism exists in *Caenorhabditis elegans*, and operates via *let*-7 family of microRNAs (see Hammell et al., 2009; Schaedel et al., 2012). As *let-7* homologues have not yet been identified or characterised in *H. contortus*, further work is required to establish how this feedback mechanism works in this parasitic nematode. Interestingly, Δ4-DA was not detected in *H. contortus*, which might be due to its absence or undetectable levels in the larval stages studied.

Different "signal intensity thresholds" of DA might be required for larval activation versus development; we found that 100 μM of (25S)-Δ7-DA did not induce exsheathment (although there is a possibility that DA does not penetrate the L3 sheath), but did significantly stimulate larval development following exsheathment. The specific inhibition of DAF-9 (cytochrome P450) with dafadine A resulted in a significant reduction of both endogenous Δ7-DA levels and larval exsheathment/development, which could be partially reversed through the supplementation of an excess (1.25 μM) of exogenous (25S)-Δ7-DA. These findings are distinct from those described for *Ancylostoma caninum* in that (25S)-Δ7-DA can directly activate infective larvae (L3s) of *A. caninum* and can induce post-parasitic larvae of *Strongyloides stercoralis* to develop to free-living stages (see Wang et al., 2009; Albarqi et al., 2016). The distinct responses to (25S)-Δ7-DA among *H. contortus* (clade V), *A. caninum* (clade V) and *Strongyloides stercoralis* (clade IV) might relate to evolutionary divergences in DA-associated signalling pathways within the phylum Nematoda (cf. Stoltzfus et al., 2012; Blaxter and Koutsovoulos, 2015). This proposal warrants future evaluation.

Exogenous (25S)-Δ7-DA-induced changes in mRNA, protein and lipid profiles in xL3s of *H. contortus* appear to link to phenotypic distinctiveness (development) and lipid metabolism (i.e., fatty acid degradation, and glycero- and glycerophospho-lipid biosynthesis) via the DA-DAF-12 module. To test the functionality of the DA-DAF-12 module, we blocked the biosynthesis of Δ7-DA using a specific inhibitor (i.e., dafadine

A) of DAF-9 (cytochrome P450) (Luciani et al., 2011), which resulted in a reduction of the endogenous Δ7-DA level, and, consequently, inhibited larval development. Similar results were achieved when the cytochrome P450s of *Nippostrongylus brasiliensis* and *S. stercoralis* were targeted with a less specific inhibitor, ketoconazole (Huang et al., 2010; Albarqi et al., 2016). The significant reduction of PC(15:0_20:4, 16:0_17:0), LPC(15:0) and PI(15:0_20:4) levels in dafadine A-treated *H. contortus* larvae could be reversed by supplementation with (25S)-Δ7-DA, indicating a direct or indirect role for DA-DAF-12 signalling in the metabolism of selected glycerolipids and glycerophospholipids (Burton et al., 2018). In addition, the increases in DG(15:0_18:1) and TG(15:0_10:0_18:2) levels in dafadine A-treated worms suggested a role for DAF-9 in glycerolipid metabolism, in accord with results for *Caenorhabditis elegans* (see Wang et al., 2015). Interestingly, all of these lipid species are odd-chain fatty acids, which contrasts the situation in *Caenorhsbditis elegans*, in which only small amounts of straight, odd-chain fatty acids (likely originating from the worm's food source - *Escherichia coli*) accumulate in lipids (Watts and Ristow, 2017). Surprisingly little is known about the origin and functional roles of these odd-chain lipid species in developmental processes of nematodes. Nonetheless, based on the present findings, we propose a dual role for the DA-DAF-12 module in promoting the metabolism of key glycerophospholipids and inhibiting the degradation of some lipids (possibly promoting fat accumulation), which functions via a negative feedback to DAF-9 (see Fig. 7.7G), but, clearly, this hypothesis requires rigorous testing.

Taken together, the findings of the present study provide evidence for a signalling cascade in *H. contortus*, in which host signals (e.g., CO_2, pH, insulin and/or metabolites of bile acids) bind to chemoreceptors, which trigger signal transduction from chemosensory neurons to endocrine cells and then hypodermal cells through the interconnected cGMP, TGF-β and IGF-1 pathways. The transduced signal promotes the metabolism of steroids and the biosynthesis of DA, the latter of which activates the nuclear hormone receptor DAF-12, leading to gene transcription and protein expression associated with body morphogenesis and pharynx development as well as lipid metabolism. A high level of DA would modulate phosphatidylinositol signalling that activates PI3K-AKT signalling (Toker and Cantley, 1997), resulting in phosphorylation-dependent cytoplasmic sequestration of the transcription factors DAF-16/FOXO (cf.

Carrivaburu et al., 2003; Hu, 2007). The activation of DAF-16/FOXO antagonises upstream cGMP, TGF-β and IGF-1 signalling (Carricaburu et al., 2003; Matsunaga et al., 2016), downregulating DA biosynthesis in a feedback circuit, resulting in a reduced lipid metabolism, and, consequently, in fat accumulation (Fig. 7.7G). Understanding the biosynthesis of DAs and nuclear-hormone signal transduction (e.g., via DA-DAF-12) should provide valuable insights into the developmental biology and adaptation of parasitic nematodes to host animals. Experimental evidence (Wang et al., 2009; Martin, 2018; Patton et al., 2018) has already shown that *Strongyloides stercoralis* hyperinfection can be prevented by treatment with (25S)-Δ7-DA. Although (25S)-Δ7-DA might regulate developmental processes in *H. contortus* (order Strongylida) differently from those in *Strongyloides* (Ogawa et al., 2009; Wang et al., 2009; Albarqi et al., 2016), the potential of DAF-9 and DAF-12 as novel intervention targets (cf. Wang et al., 2009, 2017) should be explored further. Clearly, major success achieved in a recent study (Dulovic and Streit, 2019) opens the door to assessing the functional essentiality of these steroid hormone signalling components in Strongyloides species by RNA interference.

In conclusion, current findings for *H. contortus* indicate that the hormonal signal complex DA-DAF-12 modulates the dauer-like signalling pathway through a feedback loop, and regulates biological processes associated with cellular growth and lipid metabolism via a conserved DA-DAF-12 signalling module during developmental transition. This module provides a paradigm to investigate aspects of the developmental and possibly reproductive biology of *H. contortus* and related nematodes, to explore physiochemical cross-talk between parasite and host, and to discover novel intervention strategies against parasitic diseases.

7.5. References

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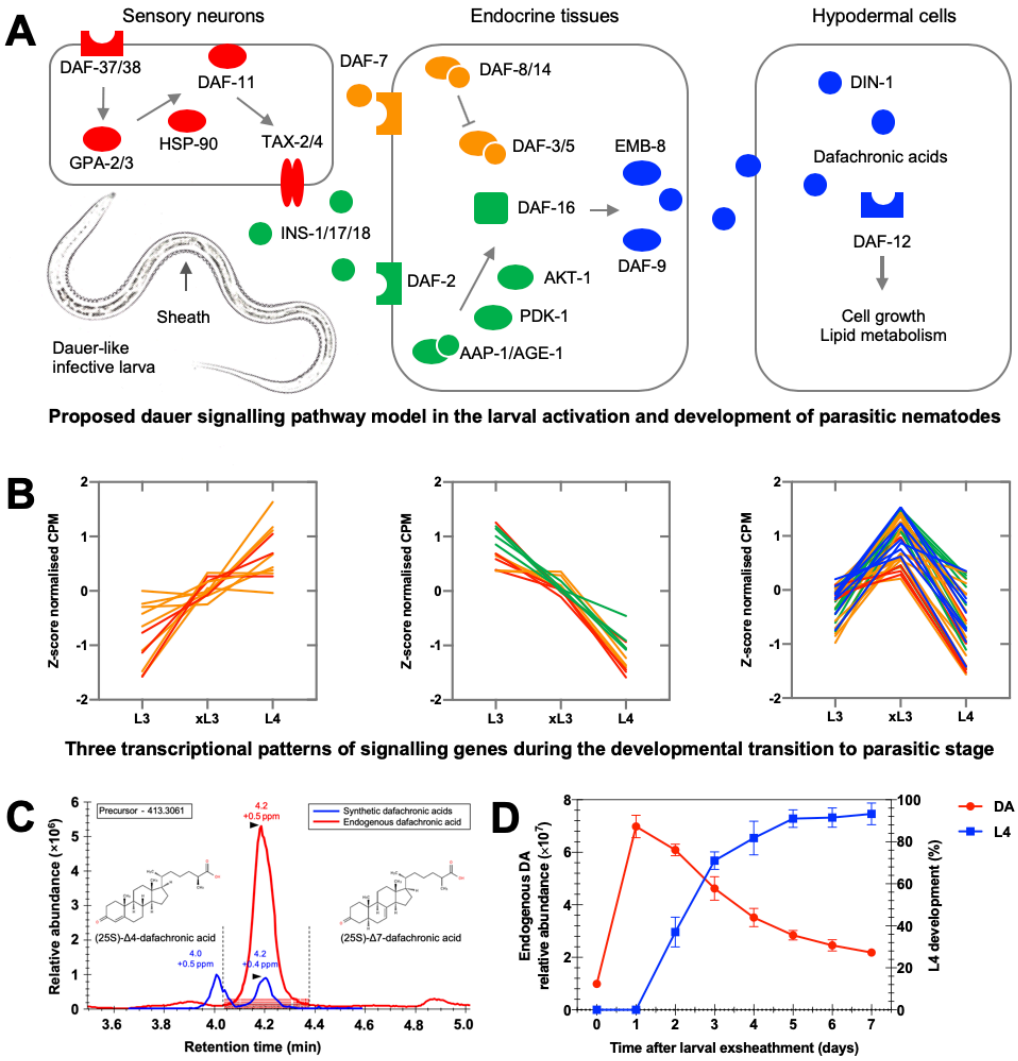


Fig. 7.1. Transcriptional changes pertaining to dauer signalling genes, and quantification of dafachronic acids in *Haemonchus contortus* during developmental transition. (A) Model of the cyclic guanosine monophosphate (cGMP) (red), DAF-7-related transforming growth factor-β (TGF-β) (orange), DAF-2-related insulin-like growth factor 1 (IGF-1) (green) and steroid hormone signalling (blue) pathways proposed for *H. contortus* (cf. Chapter 6). This model is predicted to play a role in integrating environmental signals to control the biosynthesis of one or more dafachronic acids (DAs), which activate the nuclear hormone receptor DAF-12. The DA-DAF-12 module might serve as a checkpoint for developmental decisions and associate with nutrient metabolism in parasitic nematodes (Stoltzfus et al., 2012; Wang et al., 2015; Gilabert et al., 2016). (B) Transcription profiles (Z-score normalised mapped reads per million) of 61 gene homologues involved in the cGMP (red), TGF-β (orange), IGF-1 (green) and steroid hormone (blue) signalling pathways are displayed for the developmental transition from the dauer-like third larval stage (L3), via exsheathed L3 (xL3), to the parasitic fourth larval stage (L4) of *H. contortus* in vitro. Five replicates are included for each stage. (C) Using (25S)-Δ4-DA (calculated mass. 413.3061, retention time. 4.0 min) and (25S)-Δ7-DA (calculated mass. 413.3061; retention time. 4.2 min) as references (blue peaks), endogenous Δ7-DA (retention time. 4.2 min; red peak) was detected in *H. contortus* with mass error estimated at 0.5 part per million (ppm). (D) The relative abundance of endogenous Δ7-DA following larval exsheathment and in the ensuing larval development in vitro is indicated.

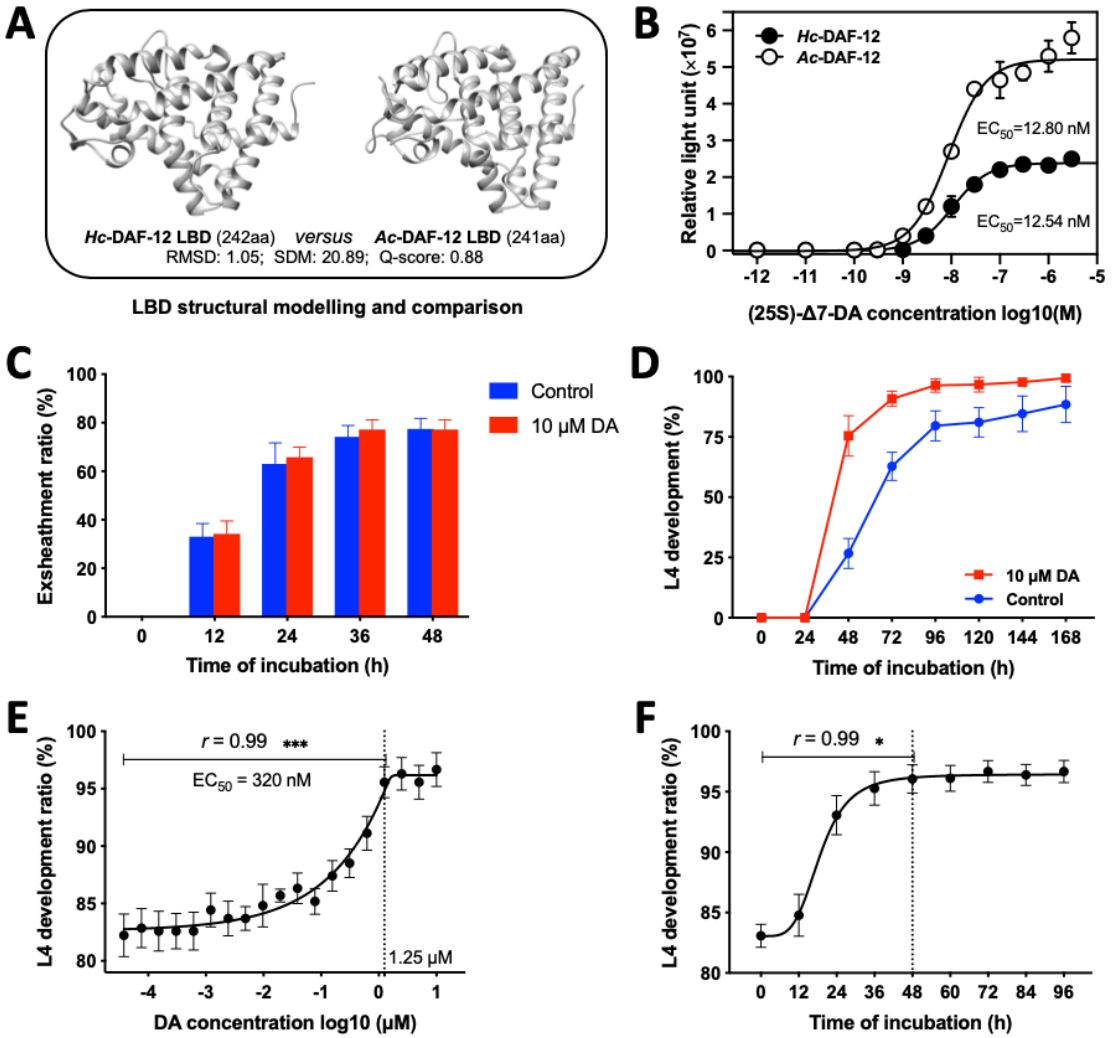


Fig. 7.2. The influence of (25S)-Δ7-DA on larval activation and development. (A) Comparison of the ligand-binding domain (LBD) of DAF-12 of *Haemonchus contortus* (Hc-DAF-12) with that of Ac-DAF-12 from *Ancylostoma caninum*, using the following parameters: sequence length, root-mean-square deviation (RMSD), structural distance measure (SDM) and Q-score. (B) Activation of *Hc*-DAF-12 and *Ac*-DAF-12 by (25S)-Δ7-DA) in a luciferase reporter assay. The effects of 10 μM of (25S)-Δ7-DA on (C) larval exsheathment and (D) larval development. The effect of (25S)-Δ7-DA on larval development is both (E) dose- and (F) time- dependent. An error bar indicates a standard deviation (SD; four replicates). Statistical significance is indicated with one or more asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, using Student's t-test).

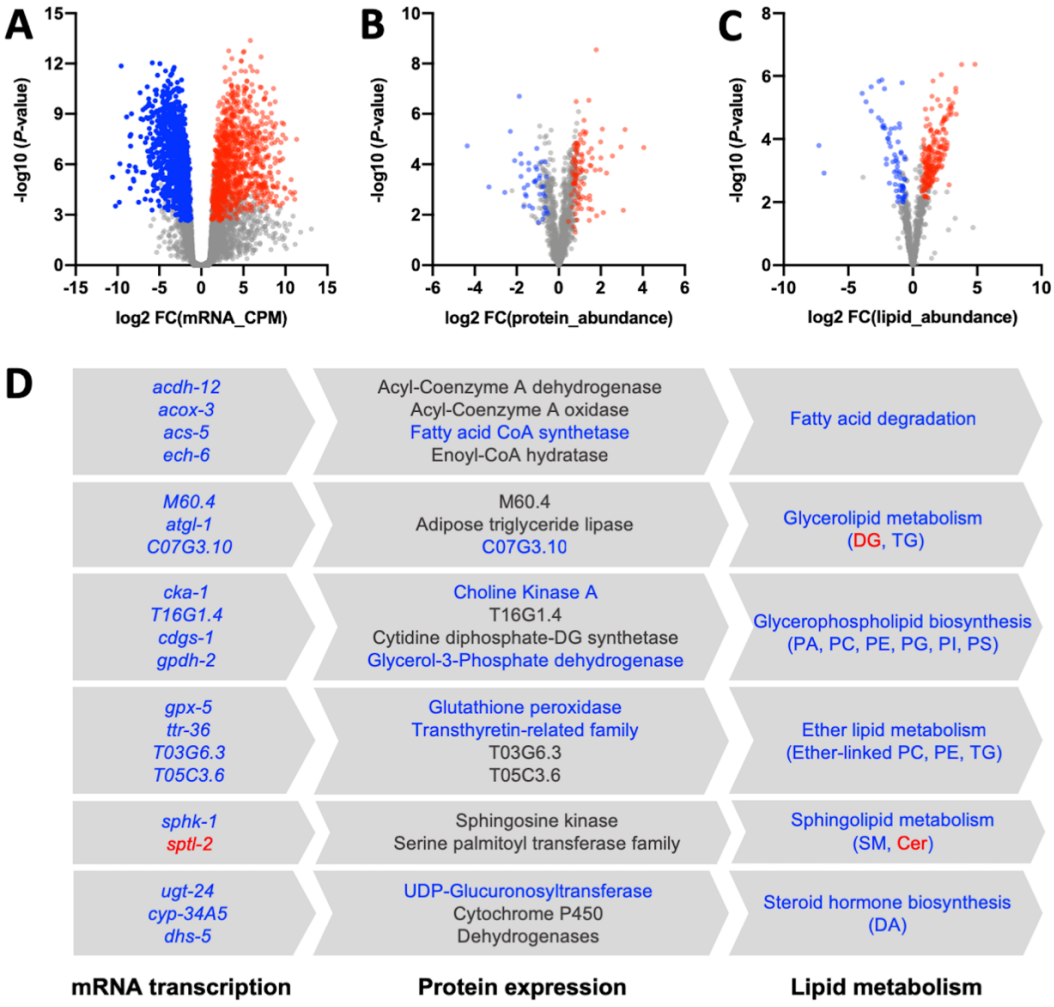


Fig. 7.3. Alterations in mRNA transcription, protein expression or lipid metabolism in *Haemonchus contortus* following larval exsheathment. Differential analyses of (A) mRNA, (B) protein or (C) lipid levels between exsheathed L3s (xL3s) at 0 h and xL3s at 24 h. Indicated is a significant up-regulation (red) or down-regulation (blue) of mRNA transcription, protein expression or lipid levels in larvae (xL3s) 24 h after exsheathment. (D) By integrating all results, we showed that molecules (mRNAs encoded by particular genes, proteins and lipids) with significant differential transcription, expression or abundance were specifically associated with fatty acid degradation, glycerolipid metabolism, glycerophospholipid biosynthesis, ether lipid or sphingolipid metabolism and/or steroid hormone biosynthesis. Down-regulated (blue) or up-regulated (red) molecules or pathways indicated; gene and protein designations derived from *Caenorhabditis elegans* homologues (WormBase).

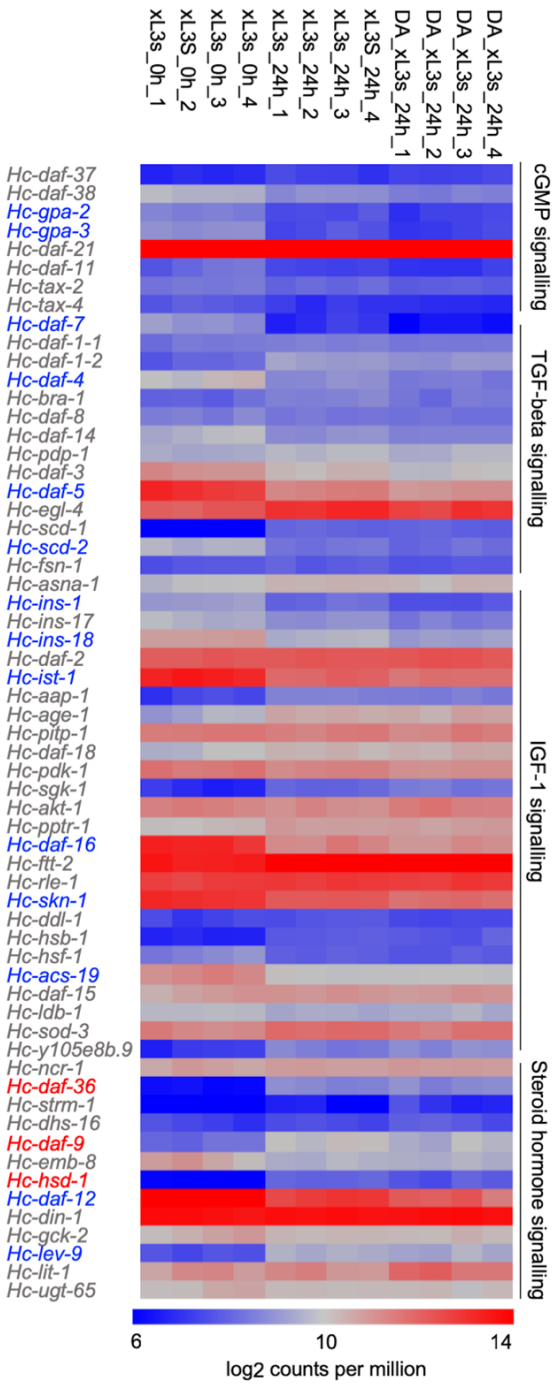


Fig. 7.4. Effect of (25S)-Δ7-DA on the transcription of dauer signalling genes following larval exsheathment. Transcription levels of dauer-like signalling genes in *Haemonchus contortus* exsheathed third-stage larvae (xL3s; 0 h and 24 h) and (25S)-Δ7-DA-treated xL3s (24 h) following exsheathment are indicated in the heat map. Genes involved in the cyclic guanosine monophosphate (cGMP), transforming growth factor-β (TGF-β) and insulin-like growth factor 1 (IGF-1) and steroid hormone signalling pathways are listed. Colour scales indicates scaled read counts per million in the rows; up-regulation (red) or down-regulation (blue) is indicated. Gene designations relate to the dauer-signalling pathway model for *H. contortus* (see Chapter 6).

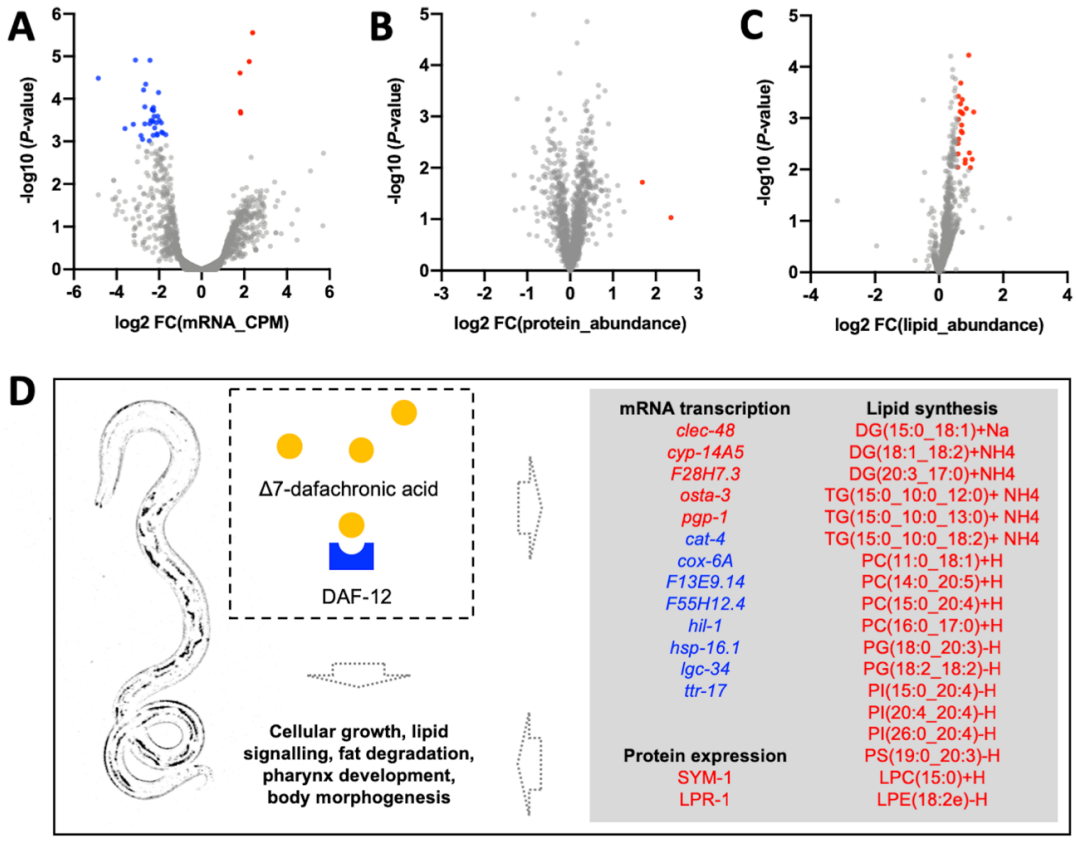


Fig. 7.5. Transcriptomic, proteomic and lipidomic differences between treated and untreated worms. Differential analyses of the (A) mRNA, (B) protein and (C) lipid levels between exsheathed the third-stage larvae (xL3s) and (25S)-Δ7-DA-treated xL3s of *Haemonchus contortus* at 24 h following exsheathment. Molecules that were significantly up-regulated (red) or down-regulated (blue) in (25S)-Δ7-DA-treated xL3s are indicated. (D) These molecular alterations inferred to associate with cellular growth, lipid signalling, fat degradation, pharynx development and body morphogenesis; gene and protein designations derived from *Caenorhabditis elegans* homologues (WormBase).

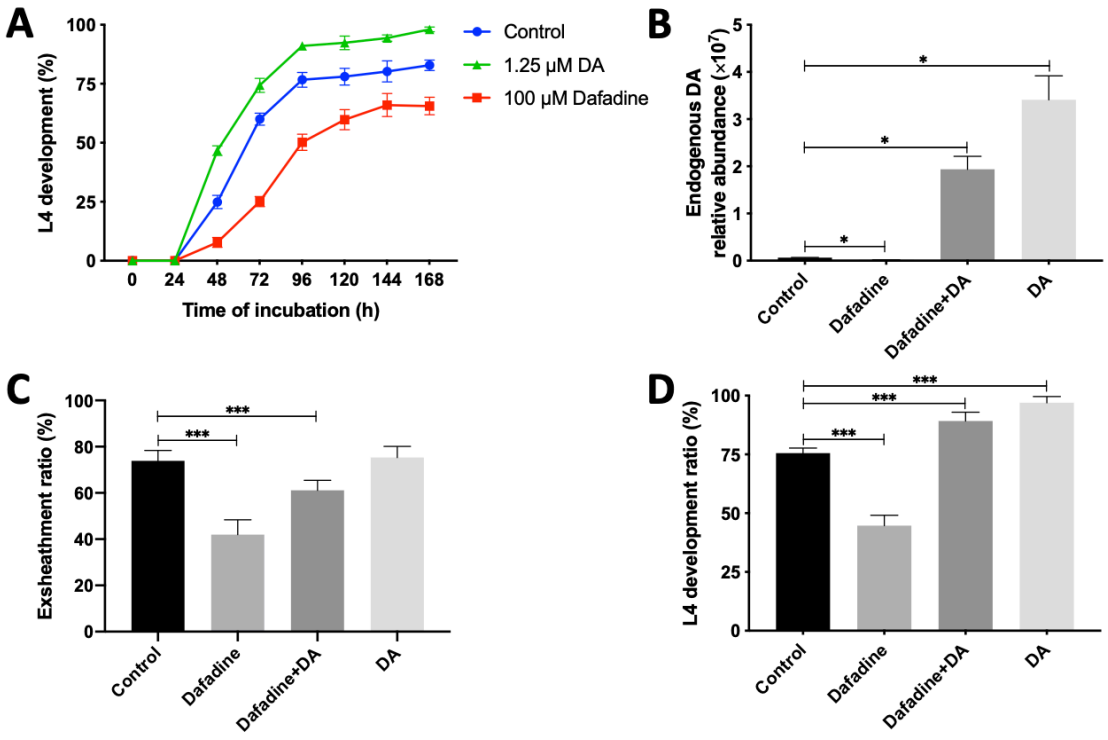


Fig. 7.6. Effects of dafadine A on DA biosynthesis, larval development and lipid metabolism. (A and B) Treatment with 100 µM of dafadine A results in a depressed larval development, which is linked to (C) a significantly lower level of endogenous Δ7-DA in dafadine-treated worms. The inhibitory effect of dafadine A and rescuing effect of 1.25 µM of (25S)-Δ7-DA on (D) larval exsheathment and (E) development. An error bar indicates a standard deviation (SD; four replicates). Statistical significance is indicated with one or more asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, using the Student's t-test).

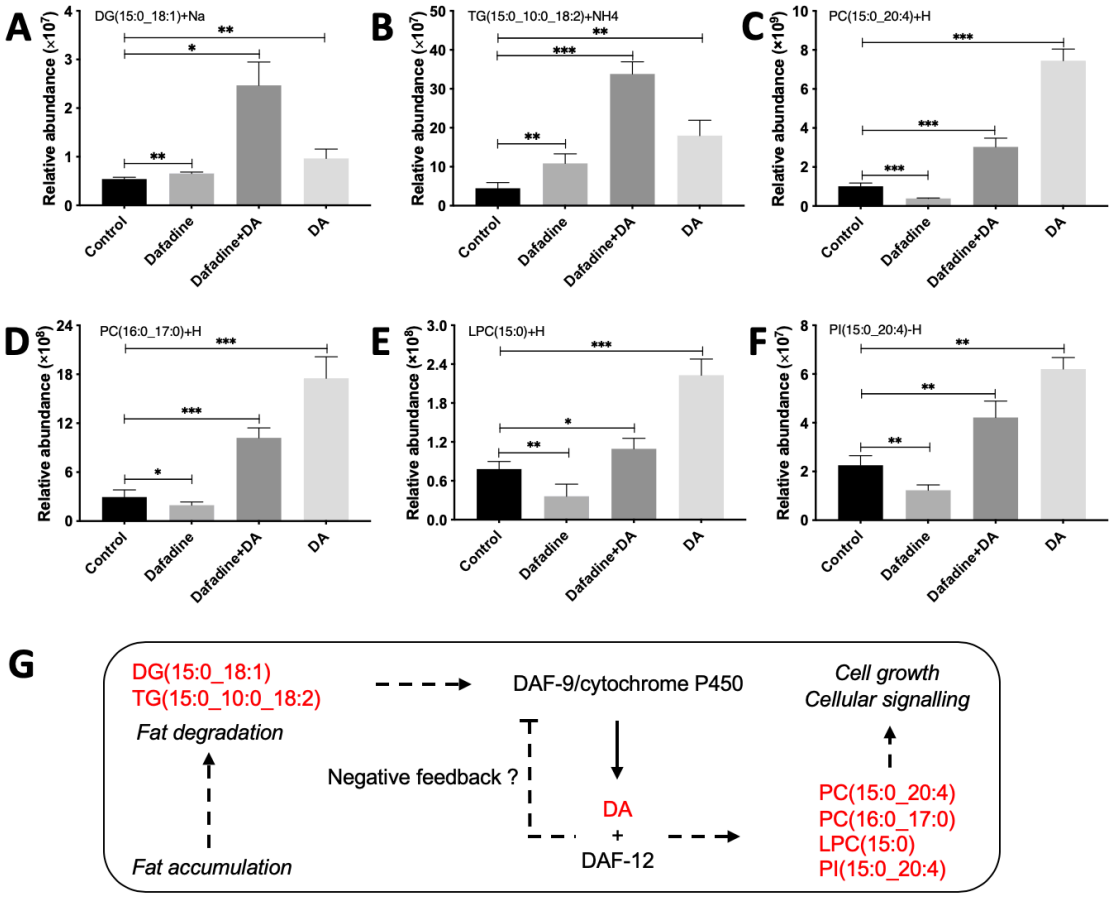
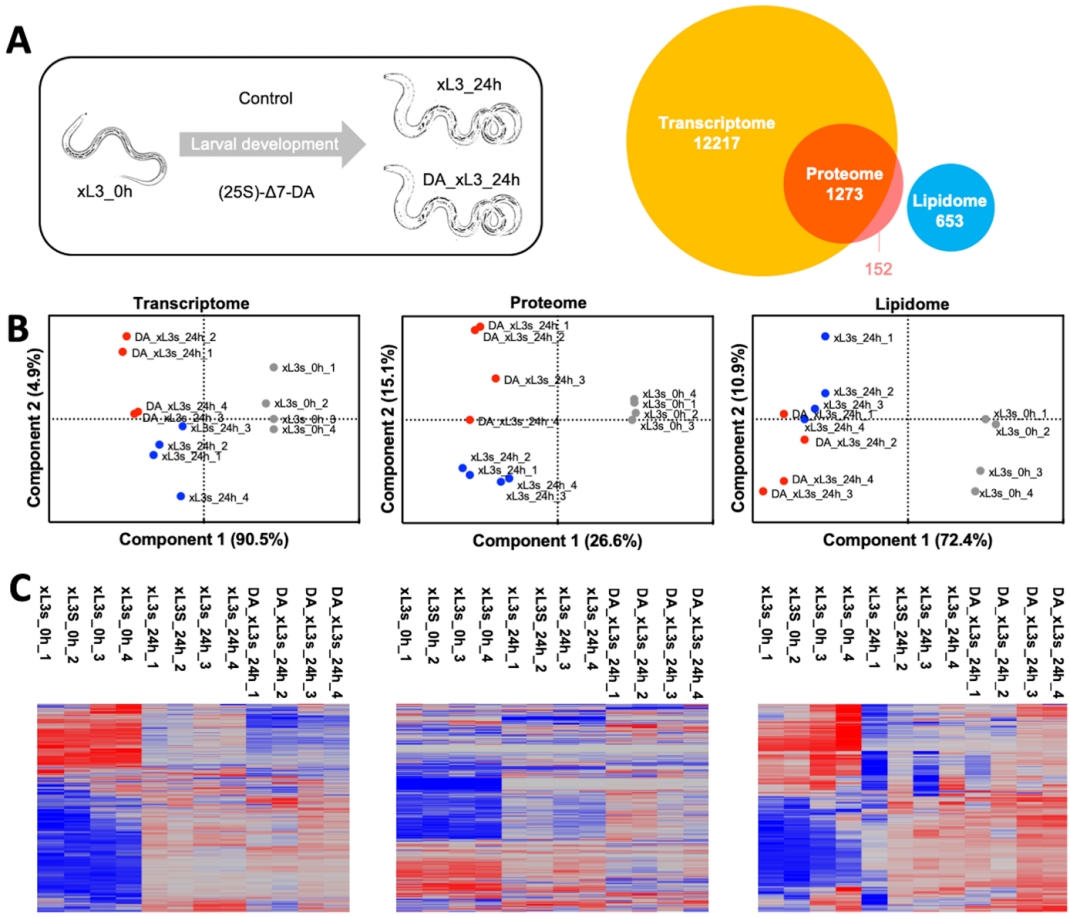
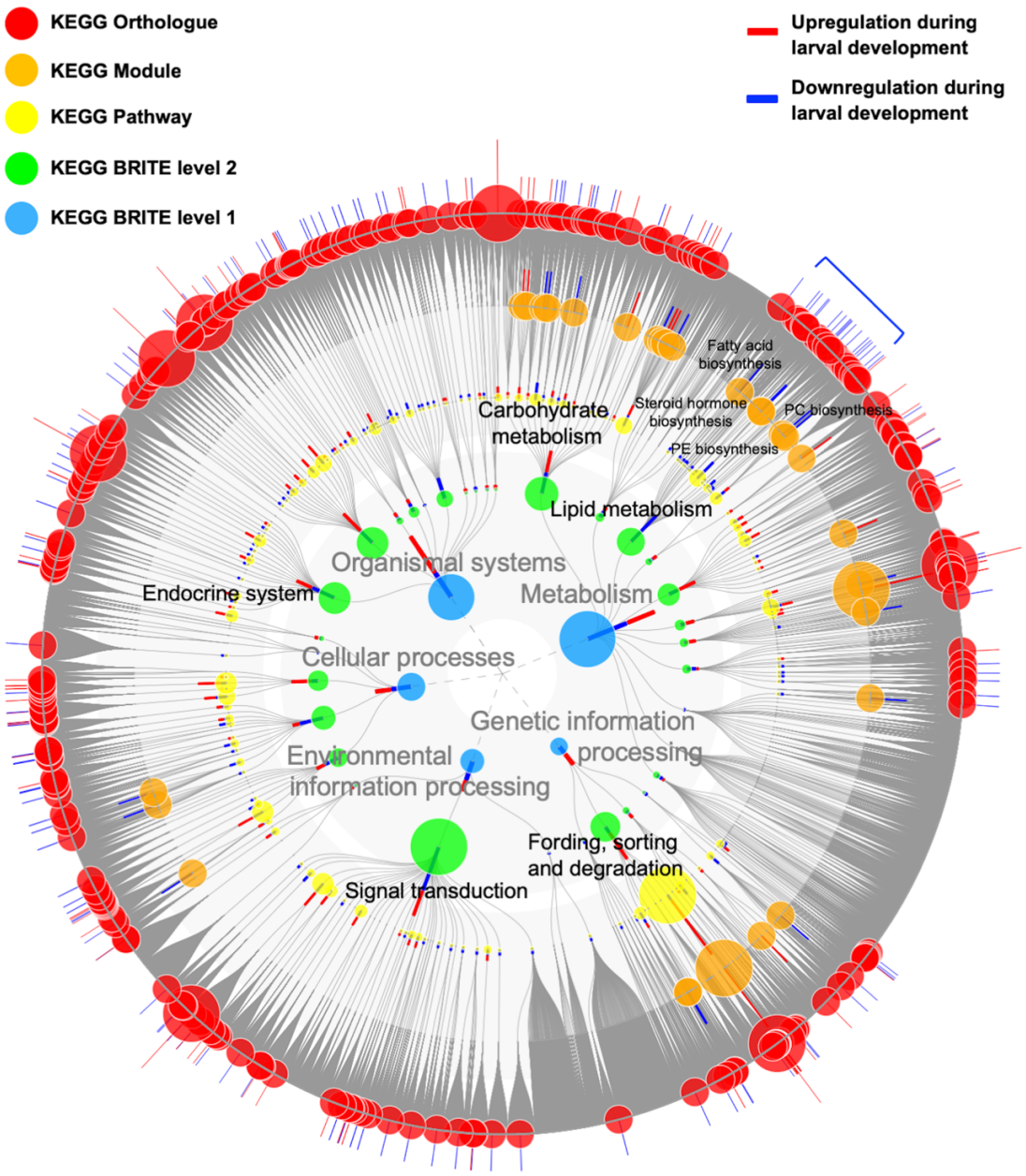


Fig. 7.7. Effects of dafadine A (100 μM) on the abundances of glycerolipids and glycerophospholipids. The abundances of particular (A) diradylglycerol (DG), (B) triacylglycerol (TG), (C and D) phosphatidylcholine (PC), (E) lysophosphatidylcholine (LPC) and phosphatidylinositol (PI) in untreated xL3s; dafadine A-inhibited xL3s; 1.25 μM of (25S)-Δ7-DA-rescued xL3s; and 1.25 μM of (25S)-Δ7-DA-treated xL3s. An error bar indicates a standard deviation (SD; four replicates). Statistical significance is indicated with asterisk (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, using the Student's t-test). (G) A schematic showing the DA-DAF-12 signalling module and its proposed functional roles in regulating fat degradation/accumulation, cell growth and cellular signalling. It is proposed that DA produced by DAF-9 (cytochrome P450) activates the nuclear hormone receptor DAF-12, which promotes the degradation of glycerolipids [e.g., DG(15.0_18.1) and TG(15.0_10.10_18.2)] for the subsequent production of glycerophospholipids [e.g., PC(15.0_20.4), PC(16.0_17.0) and PI(15.0_20.4)], and which negatively regulates DA biosynthesis to reduce lipid degradation for fat accumulation. The solid arrow indicates the production of endogenous DA; a dashed line with an arrow indicates an indirect pathway; and a dashed line with a bar indicates a negative regulation.



Supplementary Fig. S7.1. Transcriptomic, proteomic and lipidomic datasets for the exsheathed third-stage larvae (xL3s) and (25S)-Δ7-DA-treated xL3s. (A) Transcriptome, proteome and lipidome produced from xL3s (0 h and 24 h) and xL3s exposed to (25S)-Δ7-DA (24 h). (B) Principal component analyses and (C) hierarchical clustering of the transcriptomic, proteomic and lipidomic datasets.



Supplementary Fig. S7.2. Annotation and integration of transcriptomic and proteomic data sets. Functional annotation of mRNAs and proteins differentially transcribed/expressed between exsheathed L3s (xL3s) at 0 h and xL3s at 24 h. Annotation using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [(employing Orthologue (red), Module (orange), Pathway (yellow) and BRITE levels 2 (green) and 1 (blue); see Materials and methods section)]. Significantly up-regulated (red) or down-regulated (blue) molecules and pathways are indicated.

Supplementary Table S7.1. Transcription profiles of dauer signalling genes during the developmental transition from the free-living L3 stage to the parasitic L4 stage of *Haemonchus contortus* in vitro.

Supplementary Table S7.2. Effect of (25S)-Δ7-DA on gene transcription during the developmental (larval) transition of *Haemonchus contortus* in vitro.

Supplementary Table S7.3. Effect of (25S)-Δ7-DA on protein expression during the developmental (larval) transition of *Haemonchus contortus* in vitro.

Supplementary Table S7.4. Effect of (25S)-Δ7-DA on lipid abundance during the developmental (larval) transition of *Haemonchus contortus* in vitro.

Supplementary Table S7.5. Effect of (25S)-Δ7-DA on the upstream dauer signalling pathway during the developmental (larval) transition of *Haemonchus contortus* in vitro.

Chapter 8. Human toxocariasis

Abstract

Parasitic nematodes of the genus *Toxocara* are socioeconomically important zoonotic pathogens. These parasites are usually directly transmitted to the human host via the faecal-oral route and can cause substantial clinical disease, known as toxocariasis, as well as associated complications including allergic and/or neurological disorders. Although it is estimated that tens of million people are exposed to or infected with *Toxocara* species, there is limited precise epidemiological information on the relationship between seropositivity and associated disease (toxocariasis) on a global scale. Some recent studies indicate that toxocariasis is having an increased human-health impact in some countries. To gain improved insights into human toxocariasis, this article reviews salient background on *Toxocara* and biology, summarises key aspects of the pathogenesis, diagnosis and treatment of toxocariasis, describes what is presently known about its geographic distribution and prevalence, and makes some recommendations regarding future research towards the prevention and control of this important disease.

8.1. Rationale

Chapters 6 and 7 stimulated the exploration of dauer-like signalling pathways in nematodes other than *H. contortus* (clade V). The extensive genomic and transcriptomic data available publicly and in the Gasser Laboratory for selected ascaridoids "opened the door" to exploring homologous pathways in *Toxocara canis* and related worms (clade III). In order to get a better understanding of *Toxocara* and toxocariasis, I elected to review the literature in this area as a foundation for Chapter 9.

8.2. Introduction

The first diagnosis of human toxocariasis was the detection of *Toxocara* larvae in enucleated eyes from children with suspected retinoblastoma more than 60 years ago (Wilder, 1950; Nichols, 1956). Histological examination of the retinas of these diseased eyes revealed granulomatous lesions that contained larvae identified as *Toxocara canis* (Nichols, 1956). Since then, various clinical forms of human toxocariasis have been recognised: ocular larva migrans; visceral larva migrans; covert or common toxocariasis and neurotoxocariasis (Taylor et al., 1988; Finsterer and Auer, 2007; Pivetti-Pezzi, 2009). Despite the recognition of these forms or syndromes and the increasing awareness that human toxocariasis is increasing in public health significance, particularly in subtropical and tropical regions as well as in disadvantaged communities in temperate countries (Overgaauw, 1997; Hotez and Wilkins, 2009; Rubinsky-Elefant et al., 2010; Macpherson, 2013; Lee et al., 2014; Fialho and Corrêa, 2016), there are still major knowledge gaps surrounding this disease (Cooper, 2008; Hotez and Wilkins, 2009; Smith et al., 2009; Othman, 2012; Çelik et al., 2013; Fan et al., 2015; Poulsen et al., 2015; Le et al., 2016; Holland, 2017); indeed, there is evidence from recent investigations that human toxocariasis is seriously neglected, because limited attention has been paid to its prevention, treatment and/or surveillance and because it is a non-notifiable disease (Hayashi et al., 2005; Won et al., 2008; Fu et al., 2014).

A major challenge in preventing toxocariasis is the complexity of infection sources of *Toxocara* and routes of transmission, about which we still know relatively little. Nonetheless, we do know that adult worms of *Toxocara* species live in the small intestines

of various wild or domestic definitive hosts (Fig. 8.1). For instance, *Toxocara canis* infects canid hosts, including coyotes, dogs, foxes and wolves (Fig. 8.1), and *Toxocara cati* (previously *Toxocara mystax*) and *Toxocara malaysiensis* infect felids, whereas related species, such as *Toxocara vitulorum*, *Toxascaris leonina* and *Baylisascaris procyonis*, infect various carnivorous or ruminant hosts (Gibbons et al., 2001; Fisher, 2003; Schnieder et al., 2011; Macpherson, 2013). Human toxocariasis acquired from cats, and *Toxoplasma* co-infection, may be more common than previously thought (Fisher, 2003; Jones et al., 2008). Infected definitive hosts, such as dogs and cats, excrete eggs in the faeces, which then contaminate the environment and/or the hosts' hair and embryonate under suitable conditions (humidity and temperature) (Roddie et al., 2008; Overgaauw et al., 2009; El-Tras et al., 2011), remain infective for many months or even years. In addition, *Toxocara canis* larvae can undergo arrested development in the tissues of female canids and reactivate during pregnancy to infect pups through both transplacental and transcolostral routes (Burke and Roberson, 1985a; 1985b).

Eggs containing infective third-stage larvae (L3s) are accidentally ingested by humans from contaminated food, water, soil and/or utensils. In the small intestines, L3s are released from the eggs, penetrate the intestinal wall and then travel via the circulatory system to various organs, including lungs, liver, muscles and the central nervous system. Except in the definitive canid host, L3s do not mature, but can arrest in development within tissues for many years. Tissue infection evokes an inflammatory/immune response in the body that can lead to (usually non-specific) symptoms of disease, such as fever, headaches, coughing and pains (Taylor et al., 1988; Pawlowski, 2001; Rubinsky-Elefant et al., 2010). Infective eggs ingested by paratenic or transport hosts (e.g., mice, rats, rabbits, pigs, cattle or chickens; earthworms or other invertebrates) undergo a similar fate, with L3s arresting in tissues (Strube et al., 2013). Although neither in humans nor in other paratenic hosts do larvae develop to adult worms in the intestine, L3-infected tissues from paratenic hosts can act as food for humans and definitive hosts, leading to infection or disease. For instance, the consumption of infected, undercooked or raw livers has been implicated in toxocariasis (Salem and Schantz, 1992; Yoshikawa et al., 2008). Although eliminating or reducing intestinal infections in definitive hosts (dogs and cats) by chemotherapy is (likely) the most efficient way of decreasing environmental contamination and subsequent transmission to humans and other paratenic hosts, the high

prevalence of anti-*Toxocara* serum antibodies in humans in many regions studied around the world, particularly disadvantaged communities (Hayashi et al., 2005; Fu et al., 2014), indicates that this 'control' approach is not being adequately implemented. In the present article, we review salient aspects of human toxocariasis and *Toxocara* infections, emphasise key knowledge gaps and also discuss future prospects and research areas, in order to gain improved insights into what is still a neglected parasitic disease of major public health importance.

8.3. Clinical manifestations and disease

In humans, *Toxocara* larvae penetrate the intestinal mucosa and migrate to liver, lungs and other organ systems (e.g., skeletal muscle and heart) by means of mechanical disruption and protease digestion (Strube et al., 2013), with the brain and eyes being frequently affected (Finsterer and Auer, 2007; Pivetti-Pezzi, 2009). Migrating larvae are attacked by host immune responses, resulting in local inflammation associated with eosinophilia, elevated cytokines as well as specific antibodies. Although many *Toxocara* infections are subclinical in nature, human toxocariasis can manifest itself in the syndromes abbreviated as visceral larva migrans, ocular larva migrans, neurotoxocariasis, and covert or common toxocariasis (Taylor et al., 1988; Finsterer and Auer, 2007; Pivetti-Pezzi, 2009).

Visceral larva migrans is the commonest syndrome in infected people, particularly children, with clinical signs such as coughing, wheezing, myalgia, and/or cutaneous manifestations (e.g., pruritus, rash, exczema, panniculitis and vasculitis) (Despommier, 2003). Although most visceral larva migrans cases remain inapparent, lymphadenopathy, granulomatous hepatitis, nodules myocarditis, nephritis and arthritis can be observed in some people (Pawlowski, 2001; Despommier, 2003; Kuenzli et al., 2016). In addition, long-term effects, such as the development of asthma and promotion of pulmonary fibrosis, are also suspected to be associated with visceral larva migrans (Cooper, 2008).

Ocular larva migrans is common and mostly reported to occur in children between three and 16 years of age (Despommier, 2003; Pivetti-Pezzi, 2009). Uni-ocular visual impairment can occur, accompanied by chronic endophthalmitis/retinitis and/or posterior/peripheral granulomata (Despommier, 2003). The level of impairment

associates with migrating larva or death and resultant immune reactivity against the worm and location in the eye (Pivetti-Pezzi, 2009). Blindness can result from severe vitritis, cystoid macular oedema and/or tractional retinal detachment (Pivetti-Pezzi, 2009; Martínez-Pulgarin et al., 2015).

Neurotoxocariasis is rare and reported to occur mainly in middle-aged people and less in children (Deshayes et al., 2016). This syndrome relates to the migration of *Toxocara* larvae in the central nervous system and subsequent induction of meningitis, encephalitis, cerebral vasculitis and/or myelitis, usually associated with relatively non-specific clinical symptoms (e.g., fever and headache) (Finsterer and Auer, 2007; Caldera et al., 2013; Deshayes et al., 2016). Possible associations between cerebral toxocariasis and neurodegenerative disorders (e.g., seizure, schizophrenia, cognitive deficits, idiopathic Parkinson's disease and/or dementia) have also been discussed in the literature (Finsterer and Auer, 2007; Çelik et al., 2013; Fan et al., 2015). There is particular concern related to cognitive or developmental delays among socioeconomically disadvantaged children (Walsh and Haseeb, 2012). Peripheral nervous system involvement, reflected in radiculitis, inflammation of cranial nerves and/or skeletal muscles, however, is rarely recorded in humans (Finsterer and Auer, 2007).

Covert toxocariasis in children and common toxocariasis in adults is challenging to diagnose clinically because of non-specific symptoms (Taylor et al., 1988). Clinical signs, such as fever, anorexia, headache, wheezing, nausea, abdominal pain, vomiting, lethargy, sleepiness and behavioural disorders, pulmonary symptoms, limb pain, cervical lymphadenitis and hepatomegaly, might be observed in children, whereas weakness, pruritus, rash, pulmonary dysfunction, pulmonary insufficiency and/or abdominal pain can be seen mainly in adults (Walsh and Haseeb, 2014). There are also alleged links between covert toxocariasis and asthma (Mendonça et al., 2012; Li et al., 2014). These clinical features commonly associate with moderate to high anti-*Toxocara* serum antibody titres (Taylor et al., 1988; Pawlowski, 2001; Rubinsky-Elefant et al., 2010).

8.4. Diagnosis

The definitive diagnosis of toxocariasis/*Toxocara* infection in paratenic or accidental hosts can be achieved by histopathological examination, morphometric assessment of

larvae (if present) and/or the specific detection of larval DNA in/from tissue or body fluid samples by molecular means (Smith and Noordin, 2006). As serological or immunological methods alone do not allow for an unequivocal diagnosis of infection (Smith and Noordin, 2006; Poulsen et al., 2015), PCR-based tools, using genetic markers in the first and second internal transcribed spacers (ITS-1 and ITS-2 = ITS) of nuclear ribosomal DNA (rRNA) genes or selected mitochondrial (mt) genes, can assist in the specific identification of ascaridoid nematodes (irrespective of developmental stage) and/or specific diagnosis, and have been employed for epidemiological, population genetic and systematic investigations (Jacobs et al., 1997; Rai et al., 1997; Zhu et al., 1998; Zhu and Gasser, 1998; Gasser, 2006, 2013; Ishiwata et al., 2004; Li et al., 2006; Fogt-Wyrwas et al., 2007; Jex et al., 2008; Li et al., 2008; Wickramasinghe et al., 2009; Macuhova et al., 2010; Pinelli et al., 2013). Importantly, some of these molecular tools have allowed *Toxocara* species to be unequivocally characterised genetically (Gasser, 2006, 2013). They have also allowed the discovery of new (cryptic or 'hidden') species, such as *Toxocara malaysiensis* (see Zhu et al., 1998; Li et al., 2006), whose zoonotic potential and transmission pattern are presently unknown (Le et al., 2016). Moreover, recent studies have demonstrated the specific detection of *Toxocara* DNA by PCR from bronchoalveolar lavage from mice and cerebrospinal fluid from humans (Caldera et al., 2013; Pinelli et al., 2013).

As taking tissue biopsies or fluid samples is invasive and can be impractical, diagnosis of *Toxocara* infection/toxocariasis in humans has been based on the use of serological/immunological techniques (Table 8.1) (Magnaval et al., 1991; Noordin et al., 2005; Watthanakulpanich et al., 2008; Monhamad et al., 2009; Peixoto et al., 2011; Jin et al., 2013; Roldán et al., 2015; Boldiš et al., 2015; Elefant 2016), sometimes combined with imaging methods (Macpherson, 2013; Strube et al., 2013). Techniques such as enzyme-linked immunosorbent assays (ELISAs), employing *Toxocara* [*canis*] excretory/secretory (TES) antigens (de Savigny et al., 1979), have been widely used for serodiagnosis and epidemiological studies of *Toxocara* infection/exposure in humans (Boldiš et al., 2015; Moreira et al., 2014). Initial serological findings need to be confirmed by immunoblotting to avoid false-positive results and to evaluate cross-reactivity with other infective agents (Moreira et al., 2014). Thus, the combined use of ELISA and immunoblot is preferable (Moreira et al., 2014), although there is no unequivocal

serological criterion that distinguishes active *Toxocara* infection from past exposure - an area of much discussion in the field (Smith et al., 2009).

The specificity and sensitivity of serological/immunological assays for toxocariasis are dependent on both the antigens (e.g., crude products from *Toxocara canis* larvae, native or recombinant TES products, or either glycan antigens or deglycosylated TES, deTES) and the type of antibodies (e.g., total IgG, IgG subclass or IgM) being detected (cf. Table 8.1) (Roldán et al., 2015; Elefant et al., 2016). Therefore, modified TES-ELISAs, such as IgG2-TES-ELISA and IgG4-rTES-ELISA, have been developed and assessed (Noordin et al., 2005; Watthanakulpanich et al., 2008). Assays for the specific detection of IgG2 and IgG3 anti-*Toxocara* antibodies appear to achieve increased sensitivity and specificity (Magnaval et al., 1991). By contrast, an IgG4-ELISA, employing recombinant TES-120 (rTES-120) and rTES-30, was reported to have 100% sensitivity, and IgG-ELISA using deglycosylated TES was reported to increase both sensitivity and specificity to 100% (Mohamad et al., 2009; Roldán et al., 2015). Other recombinant *Toxocara* proteins are also under evaluation as potential diagnostic antigens (Anderson et al., 2015; Zhan et al., 2015). Additionally, measuring eosinophil cationic protein levels or IgG antibody avidity can be used to suggest current or past *Toxocara* infection (Hübner et al., 2001; Magnaval et al., 2001; Dziemian et al., 2008; Boldiš et al., 2015). However, the performance of each of these assays needs to be comprehensively validated in various countries under different conditions.

Particularly for the diagnosis of ocular larva migrans, specific anti-*Toxocara* antibody in serum and vitreous or aqueous humour should be assessed, while the diagnosis of neurotoxocariasis should include the detection of specific antibodies in serum as well as eosinophils in cerebrospinal fluid (CSF) (Macpherson, 2013; Caldera et al., 2013). In addition to the use of these serological or immunological methods, imaging techniques can assist clinical diagnosis in patients with ocular larva migrans or visceral larva migrans. Nonetheless, there is a need for improved diagnostic algorithms and clinical definitions for both neurotoxocariasis and covert/common toxocariasis. Various medical imaging techniques, such as ultrasound, computerised tomography and magnetic resonance imaging have been utilised to scan hepatic lesions relating to visceral larva migrans, and optical coherence tomography, fluorescein angiography, computerised

tomography and ocular ultrasound can also be used to support the diagnosis of ocular larva migrans (Macpherson, 2013; Martínez-Pulgarin et al., 2015).

8.5. Epidemiology and public health importance

Toxocara canis and *Toxocara cati* have been recognised to have a worldwide distribution (Fisher, 2003; Lee et al., 2010). However, in order to provide a relatively accurate assessment of the prevalence of *Toxocara* infections/exposure in humans, as determined using serological assays, we reviewed the literature (Fig. 8.2; Supplementary Table S8.1). Some nation-wide epidemiological investigations of *Toxocara canis* and *Toxocara cati* in dogs and cats have indicated respective prevalences of 1.2% and 3.2% in Australia (Palmer et al., 2008), 4.4% and 4.6% in the Netherlands (Overgaauw et al., 2009), 6.1% and 4.7% in Germany (Barutzki and Schaper, 2010), with higher prevalences of 51-100% in puppies and 1-45% in adult dogs and 3.2-91.0% in cats in some surveys in countries including Nigeria, Portugal, India and China (Fisher, 2003; Traub et al., 2005; Sowemimo, 2007; Dai et al., 2009; Lee et al., 2010; Waap et al., 2014). It has been estimated that there are more than 77 million dogs and 93 million cats in the USA, some of which play central roles in the spread of toxocariasis via the excretion of eggs in faeces into gardens, parks, playgrounds and sand pits, and represent a major risk factor of infection once the eggs become infective (Fisher, 2003; Manini et al., 2012). Humans acquire *Toxocara* infection(s) via a range of routes, such as accidental ingestion of infective eggs from contaminated soil and water, contaminated raw vegetables or fruit (Dubná et al., 2007; Poeppl et al., 2013). Another risk factor is human contact with dogs or cats (Poeppl et al., 2013), as embryonated eggs have been found on the hairs of these definitive hosts (Roddie et al., 2008; Overgaauw et al., 2009; El-Tras et al., 2011). However, the most significant observation that emerges from the work undertaken so far is the very low number of embryonated eggs detected on host hair, particularly among well-cared-for dogs (Keegan and Holland, 2010). In addition, people can be infected by ingesting encapsulated L3s in raw or undercooked meat or organs from paratenic hosts, such as rabbits, sheep, cattle and chickens (Salem and Schantz, 1992; Taira et al., 2004; Yoshikawa et al., 2008; Dutra et al., 2014). The apparent broad distribution of *Toxocara* and the various possible transmission routes indicate a relatively high risk of human

infection and that toxocariasis is a common zoonotic helminth infection (Fig. 8.2; Supplementary Table S8.1).

Nation-wide surveys of humans in the past 36 years have estimated the prevalences of specific anti-*Toxocara* serum antibody at 0.7% in New Zealand, 1.6% in Japan, 2.4% in Denmark, 6.3% in Austria, 7.0% in Sweden, 14.0% in the USA and 31.0% in Ireland, but are greater than 20% in some ethnic and socioeconomically disadvantaged groups, 22.0% in Iran and 81.0% in Nepal (Supplementary Table S8.1). Notably, a seroprevalence of 85.0% has been recorded in school children in Manado, Indonesia (Hayashi et al., 2005), and 87.0% in the Marshall Islands (Fu et al., 2014). However, the present review of the literature reveals that there is still limited detailed epidemiological information for most countries around the world.

Although human toxocariasis is expected to be common, particularly in communities where there is a close association between humans and wild or domestic canids/felids, it is challenging to assess the global impact of this enigmatic parasitic disease (Hotez and Wilkins, 2009; Smith et al., 2009), because of the limitations of existing diagnostic tools (Fillaux and Magnaval, 2013; Moreira et al., 2014). The large-scale national survey by Won and colleagues (2008) is exemplary, in that representative samples and a defined detection technique (e.g., sample dilution, immunoassay, and statistical analysis) were used to obtain seroprevalence data. This study showed that the age-adjusted seroprevalence for toxocariasis was 13.9%, and was higher in non-Hispanic blacks (21.2%) than both non-Hispanic whites (12.0%) and Mexican Americans (10.7%), suggesting a link to ethnicity. The authors highlighted that the striking differences in seroprevalence could be used to target health education messages. Nevertheless, using serological methods, it is still challenging to distinguish infections of, or exposure to, *Toxocara canis* and *Toxocara cati* (Fisher, 2003; Poulsen et al., 2015), which hinders a better understanding of the epidemiology of human toxocariasis. Overall, there is an urgent need for studies to assess the global burden of human toxocariasis, in order to better justify investments in large-scale detection and prevention programs.

8.6. Treatment

The two major obstacles to the successful treatment of toxocariasis in humans are: (a) the requirement for drugs to reach larvae across a range of somatic tissues, and (b) the difficulty in verifying drug efficacy in patients. Nevertheless, treatment with anthelmintics is advocated for acute toxocariasis, particularly to prevent *Toxocara* larvae from reaching the brain (including eyes) (Pawlowski, 2001; Wiśniewska-Ligier et al., 2012). Albendazole and mebendazole are commonly used for the treatment of visceral larva migrans, despite their limited efficacy against larvae in tissues (Caumes, 2003); albendazole is preferable due to its widespread distribution (of a metabolite) in tissues compared with mebendazole which is not absorbed outside of the gastrointestinal tract. In addition to anthelmintics, anti-inflammatory compounds (e.g., corticosteroids or nonsteroidal anti-inflammatory drugs, *NSAIDs*) can be given to relieve symptoms caused by allergic responses (Despommier, 2003). Specifically, although ocular larva migrans can be treated with corticosteroids (e.g., prednisolone) and, in selected/severe cases, ophthalmologic surgery (Despommier, 2003), the efficiency of treatment of ocular larva migrans is unclear, even though a combination of albendazole and steroids has sometimes resulted in favourable clinical outcomes (Barisani-Asenbauer et al., 2001; Martínez-Pulgarin et al., 2015). In addition, albendazole is recommended for the treatment of NT due to its ability to cross the blood-brain barrier, and has a better tolerability than mebendazole and diethylcarbamazine (Othman, 2012). However, there is an urgent need for larger and well-defined clinical studies, particularly in children. Moreover, despite being serologically negative, some children with toxocariasis can have symptoms, such as headaches, which persist even after three rounds of treatment with albendazole or mebendazole (Wiśniewska-Ligier et al., 2012). Efforts have been made to increase the effectiveness of the delivery of these anthelmintics to tissues, particularly the brain, by using polyethylene glycol (PEG)-conjugated and liposome-encapsulated compounds (Horiuchi et al., 2005; Hrckova et al., 2007; Barrera et al., 2010; Moreira et al., 2014).

Drugs and their formulations for the treatment of toxocariasis are summarised in Table 8.2. In particular, fenbendazole has been shown to have a relatively high efficacy at killing *Toxocara canis* in muscles (89.5%) and the brains (66.1%) of C57BL/6 mice when delivered by liposomal carrier and administrated with an immunostimulatory glucan

(Hrckova et al., 2007), and albendazole-PEG has been shown to achieve a complete kill of larvae in the brain (Leonardi et al., 2009). In addition to these drugs, the potential of some other chemicals and natural products has been discussed or studied. For instance, the larvacidal or larvistatic activities of nitazoxanide, tribendimidine, phenazines (e.g., lapachol, β-lapachone, and β-C-allyl-lawsone), and fatty acid amides (e.g., linoleylpyrrolidilamide) have also been discussed (Martínez-Pulgarin et al., 2015; Mata-Santos et al., 2015). Some natural products, such as the plant extracts from *Chenopodium ambrosioides* and an aqueous extract of the nutritional supplement Nutridesintox®, have some anthelmintic activity against *Toxocara canis* larvae in vitro and a reduction in the inflammatory infiltrates in the liver and lung of CD-1 mice (Reis et al., 2010). However, such products need to undergo rigorous assessment in vivo to ensure their efficacy and safety and, if possible, to establish their pharmacodynamics.

8.7. Immunology and prospect for vaccines

There is increasing interest in veterinary vaccines against helminth infections and specifically for vaccines to prevent transmission of zoonotic diseases to humans. A recent example is the successful evaluation of a vaccine against *Taenia solium* in pigs to prevent human cysticercosis (Garcia et al., 2016). Like many other helminthic diseases, however, protective immunity is often forestalled by the parasites' ability to block and evade the host immune system (McSorley and Maizels, 2012). For *Toxocara* species, immune evasion is mediated by surface coat and excretory/secretory (ES) molecules (Maizels, 2013), which are also of interest as vaccine targets for protective type 2-like immune responses (Loukas and Maizels, 2000; Długosz et al., 2015). TES components, first explored by Maizels and colleagues (1984), and since characterised in more detail, include the C-type lectins TES-32 and -70 (Loukas et al., 1999, 2000; Loukas and Maizels, 2000) and heavily glycosylated mucins which also make up the surface coat of the larval parasite (Kennedy et al., 1987; Gems and Maizels, 1996; Loukas et al., 2000; Maizels et al., 2000; Długosz et al., 2015). Recently, based on analyses of the genome and transcriptomes of *Toxocara canis* (Zhu et al., 2015), it is proposed that a wider range of peptidases, adhesion molecules, SCP/TAPS proteins and lectins also play roles in the host-parasite interplay (Gasser et al., 2016).

Major features of the host immune response to *Toxocara* infection include a dominant $CD4^+$ T-helper type 2 cell (Th2) activity, eosinophilia and production of specific antibodies (Del Prete et al., 1991; Maizels, 2013). TES antigens drive a vigorous production of type 2 cytokines (e.g., interleukin-4, IL-5, -10, and -13) from peripheral T cells of exposed individuals, resulting in eosinophilia, enhanced cytokine expression and the production of IgE antibodies (Del Prete et al., 1991; Maizels, 2013; Mazur-Melewska et al., 2016). These factors can all contribute to airway hypersensitivity, linking chronic *Toxocara* infection with allergic diseases, such as asthma and allergic rhinitis (Yariktas et al., 2007; Copper, 2008). Interestingly, wheezing in people can be associated with visceral larva migrans and covert/common toxocariasis due to pulmonary migration of *Toxocara* larvae (Taylor et al., 1988; Mendonça et al., 2012). Whether these subjects, mostly children, develop asthma later in their childhood remains controversial – there is a possibility that they might be protected from atopic asthma as a result of the immunoregulatory responses induced by infection (Cooper, 2008; Maizels, 2013). Although many parasitic helminths can induce immunoregulatory cell populations (e.g., regulatory T cells and alternative-activated macrophages) (Hewitson et al., 2009), it is likely that *Toxocara canis* has evolved mechanisms effective in the dog rather than humans, explaining its greater immunogenicity in the accidental host. Nevertheless, some anti-inflammatory effects can be mediated by TES, resulting, for example, in the inhibition of Toll-like receptor signalling and nitric oxide (NO) production (Hewitson et al., 2009).

Immunomodulation is a feature of *Toxocara* infections in humans and other hosts. For example, increased protective pro-inflammatory cytokines (IL-6, IFN-gamma and IL-13) and anti-inflammatory cytokine (IL-10) can be observed in sera from *Toxocara*-infected children (Nagy et al., 2012), and macrophages from infected mice show a shift towards greater IL-10/TGF-β and lower IL-12/TNF production (Kuroda et al., 2001). There are various mechanisms under the simultaneous activation and suppression of immune responses. Firstly, the surface-coat shedding strategy of the parasite enables migrating larvae to escape from eosinophils that adhere to the parasite surface, thus neutralising the antibody-dependent elimination (Fattah et al., 1986). Secondly, TES antigens, a Th2-stimulator, can also modulate local and systematic immune responses (Maizels, 2013). For example, C-type lectins, particularly TES-32 and TES-70, have considerable

homology to low affinity IgE and macrophage mannose receptors in mammals (Loukas et al., 1999), which are capable of targeting host pathways involved in innate immunity (Hewitson et al., 2009). Additionally, exosomes and other extracellular vesicles might also represent other means of *Toxocara* being able to modulate immune responses, because such vesicles have been shown to transfer ES antigens and small RNAs to host cells (Buck et al., 2014). However, this new and exciting area needs to be explored in much more detail. Clearly, an improved understanding of specific immune responses and modulatory mechanisms during toxocariasis as well as defining immunogenic molecules could assist in developing an effective anti-*Toxocara* vaccine.

8.8. Prevention and control

The remarkably broad distribution of *Toxocara*, its multiple infection routes (e.g., human-dog contact, neonatal and food-borne transmission), and potential associations of toxocariasis with allergic and neurological disorders have raised considerable public concern. However, we believe that community awareness of toxocariasis is still inadequate. Enhancing education will be essential to improve the public's understanding of toxocariasis, and its prevention, treatment, and control (Fig. 8.3) (Holland, 2017). To this end, web-based educational sites are currently important resources, such as the American Association of Veterinary Parasitologists (AAVP, http://www.aavp.org/) and Centers for Disease Control and Prevention (CDC, http://www.cdc.gov), and provide detailed information on *Toxocara*/toxocariasis. Individually, practicing veterinarians also need to take responsibility to educate pet owners about the importance of toxocariasis and how to minimise risks of zoonotic transmission (Smith et al., 2009), although, interestingly, veterinarians themselves are commonly infected/exposed due to their occupational contact with small animals (Poeppl et al., 2013; Deutz et al., 2005). Among physicians and other human healthcare providers, there is almost no knowledge base of toxocariasis, such that efforts to diagnose and test children and adults with asthma-like symptoms and pulmonary dysfunction (covert/common toxocariasis) or cognitive deficits (neurotoxocariasis) are almost non-existent. Hygiene, preventing children from ingesting infective *Toxocara* egg-contaminated soil or faeces from carnivores as well as avoiding the ingestion of raw or uncooked meat or liver are central to preventing transmission in

endemic countries (Fan et al., 2013; Moreira et al., 2014). Moreover, although asymptomatic forms of this disease are usually self-limiting, any case of human toxocariasis should be treated to prevent larvae from invading the brain, including eyes (Pawlowski, 2001; Othman, 2012; Wiśniewska-Ligier et al., 2012).

The relationship among humans, animals and the environment should always be kept in mind. Since there is a wide range of reservoirs and various transmission routes available to *Toxocara* species, a comprehensive one-health approach should be used for effective control of human toxocariasis (Fig. 8.3) (Holland, 2017). Controlling *Toxocara* infection in definitive hosts should be a priority to substantially reduce the number of infective eggs in the public environment (Palmer et al., 2008). Pet dogs and cats, particularly puppies of < 12 weeks of age and kittens (Morgan et al., 2013; Nijsse et al., 2014), as well as working dogs, and, where possible, stray dogs and cats should be dewormed with effective anthelmintics (Roddie et al., 2008; El-Tras et al., 2011). However, an aspect that should be kept in mind is that anthelmintic treatment of pregnant dogs and cats is mostly ineffective at preventing transplacental and/or transmammary transmission (Overgaauw and van Knapen, 2013). Moreover, interventions to prevent dog fouling have also been suggested (Atenstaedt and Jones, 2011). Importantly, the intervention scenarios described by Nijsse and colleagues (2015) indicate that deworming strategies (four times per year) and the removal of faeces would reduce eggs output and resultant environmental contamination. Nonetheless, due to major knowledge gaps in the epidemiology of *Toxocara*, controlling transmission is considerably more challenging when wild animals (e.g., foxes) are involved (Morgan et al., 2013; Holland, 2017). The debate on public health measures that could be implemented in endemic settings - mostly underprivileged, rural communities and poor urban-dwelling subjects in tropical cities with poor sanitation and large stray dog or cat populations – needs to be centred not only around understanding parasite biology and epidemiology but also on the feasibility and cost-effectiveness of the measures as well as an understanding of populations at increased risk of infection (e.g., age groups, rural versus urban, and socioeconomic status).

8.9. New molecular insights using genomic and transcriptomic tools

The recently completed *Toxocara canis* genome project represents an important step toward a better understanding of this parasite and toxocariasis at the molecular and biochemical levels, and could assist in developing a next generation of diagnostic and intervention methods (Zhu et al., 2015; Gasser et al., 2016). The draft genome (317 Mb) was predicted to encode 18,596 genes, with 14,583 (78.4%) genes annotated, and with 5406 (29.1%) genes having homologues in known biological (KEGG) pathways (Zhu et al., 2015; Gasser et al., 2016). Specifically, 870 ES proteins (including proteases, cell adhesion molecules, lectins, SCP/TAPS proteins, and mucins) were predicted to be involved in host invasion and in parasite-host interactions, such as immune evasion and/or immune modulation, whereas 458 protein kinases, 408 phosphatases and 127 GTPases were proposed to have important roles in embryonic, larval development and reproduction, and 156 GPCRs, 268 ion channel proteins and 530 transporters having potential as drug targets. Additionally, intestine-, stage-, and gender-enriched molecules/pathways were defined, indicating essential involvement in survival, development and/or reproductive processes (Zhu et al., 2015; Gasser et al., 2016). The genomic data provide resources for future investigations of the immunobiology, epidemiology, genetics, and pathogenesis of *Toxocara canis*/toxocariasis as well as for the design of improved diagnosis tools and new interventions, including anthelmintics and vaccines.

In addition, since microRNAs (miRNAs) are being increasingly recognised for their roles in development, reproduction and host-parasite interactions as well as drug resistance in parasitic helminths, a recent study explored transcription profiles of small RNAs in adult *T. canis* employing the newly established genomic and transcriptomic resources (Ma et al., 2016). The findings of this study suggest that miRNAs *Tc*-miR-2305 and *Tc*-miR-6090 are likely involved in reproduction, embryo and larval development of *Toxocara canis*, whereas *Tc*-let-7-5p, *Tc*-miR-34 and *Tc*-miR-100 appear to be associated with host-parasite interactions. Moreover, some other miRNAs, including *Tc*-miR-2861, *Tc*-miR-2881 and *Tc*-miR-5126, might be drug targets and/or associated with drug resistance. Such studies are now starting to broaden and deepen our knowledge of fundamental molecular aspects of *Toxocara canis* biology, should provide a basis for

experimental investigations of the developmental biology, parasite-host interactions and disease, and might assist in the design of novel diagnostic and therapeutic approaches (Manzano-Román et al., 2012; Hoy et al., 2014; Britton et al., 2015).

8.10. Future prospects and conclusions

Although branded as "America's commonest neglected infection of poverty" (Hotez and Wilkins, 2009; Woodhall et al., 2014), the global significance of toxocariasis remains to be critically assessed (Hotez and Wilkins, 2009; Macpherson, 2013). So far, there is no estimate of the global disease burden of toxocariasis, and while there are epidemiological studies and reports of toxocariasis from all over the world, there are major knowledge gaps in the epidemiology of *Toxocara canis* and *Toxocara cati* infections as well as the more recently described *Toxocara malaysiensis*, whose zoonotic importance is presently unknown, with considerable limitations in evaluating the public impact of toxocariasis. For instance, it is challenging to integrate seroprevalence data for a number of reasons, such as the way in which populations were sampled, different detection methods utilised and differences in the quality of antigens and in the cut-off values used therein (Holland, 2017). The problems with sensitivity and specificity (cross-reactivity) of some serodiagnostic tools likely leads to false-positive results, particularly in tropical areas, where polyparasitism is common, and different serological methods can produce discrepant results (Fillaux and Magnaval, 2013; Moreira et al., 2014). Moreover, it is not possible to unequivocally differentiate *Toxocara canis* from *Toxocara cati* (or *Toxocara malayensis*) infections using existing serological methods (Schabussova et al., 2007; Poulsen et al., 2015). Therefore, optimized, species-specific diagnostic methods are needed to support future epidemiological investigations.

Equally needed are studies to understand the pathogenesis and natural history of covert/common toxocariasis and neurotoxocariasis. Potentially, these two conditions are linked to major causes of pulmonary dysfunction and cognitive delays, respectively, especially in children and adults living in poor and underserved areas, but the evidence base is still missing. Currently, we are faced with a "chicken and egg situation" regarding covert/common toxocariasis and neurotoxocariasis. By this, we mean that without the evidence base in hand, it is difficult to secure research funds to investigate these

conditions, and, yet, there are very few funded studies underway to establish any links to human toxocariasis.

Expanding our understanding of the fundamental biology, particularly immune recognition and evasion of *Toxocara* larvae, should be a priority, and could lead to new diagnostic tools and methods of intervention. Although genes expressed by the arrested infective larvae have helped us gain some insight into larval survival and immune evasion, there is still a paucity of knowledge as to how *Toxocara canis* suppresses host immunity at the molecular level and how protective immunity is achieved in definitive and paratenic hosts. The draft genome will assist investigations of the immunobiology of *Toxocara canis* as well as the genetics and epidemiology of this parasite (Zhu et al., 2015; Gasser et al., 2016). Genomic as well as transcriptomic resources should now facilitate studies of this pathogen's biology, biochemistry, physiology and processes or mechanisms involved in blocking or evading host immune attack (Gasser et al., 2016). Also novel diagnostic and therapeutic approaches could be facilitated using these resources. Since a broad spectrum of ES proteins was predicted from transcriptomic data (Zhu et al., 2015; Gasser et al., 2016), it should be possible to classify and explore TES antigens in much more detail. Since the potential of microRNAs as biomarkers for diagnosis has been discussed (Manzano-Román et al., 2012), and assessed for the diagnosis of *Brugia pahangi*, *Dirofilaria immitis*, *Onchocerca volvulus* and *Onchocerca ochengi* infections (Hoy et al., 2014; Tritten et al., 2014; Quintana et al., 2015), there seems to be promise in identifying specific biomarkers for the diagnosis of toxocariasis.

Genomic-guided methods might complement conventional approaches for drug and drug target discovery (Zhu et al., 2015; Gasser et al., 2016). For example, eight recognised targets (e.g., SLO-1 calcium-activated potassium channels, acetylcholine receptors) and 101 potential targets (e.g., kinases and phosphatases) were predicted from *Toxocara canis*, which might be tested for their essentiality by gene silencing in *Toxocara canis*. The gene silencing machinery of *Toxocara canis* has been characterised, with 43 RNA mediated interference (RNAi) effector genes being predicted to date (Zhu et al., 2015). Although the uptake of extracellular double-stranded RNAs (dsRNAs) might be limited due to the lack of the *sid-2* gene, previous gene knock-down analyses in *Ascaris suum* and *Toxocara canis* suggest promise for functional genomic investigations of parasitic-specific genes in *Toxocara canis* (Ma et al., 2015; McCoy et al., 2015). In addition, small

RNA technology might assist in the development of novel therapeutics and vaccines (Britton et al., 2015). In conclusion, human toxocariasis is a common, neglected parasitic disease of global importance, but remains enigmatic in many respects. Increased public awareness is essential for the treatment and control of this disease. The availability of new molecular resources of *Toxocara* species and advanced molecular tools now underpins toxocariasis research and should facilitate the development of radically new diagnostic and intervention strategies.

8.11. References

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Table 8.1. Summary of key serological techniques employed for the detection of anti-*Toxocara* antibodies, and a description of their purpose and diagnostic performance.

Technique*	Purpose and performance	References
IgG-TES-WB	Detection of specific total IgG in human sera, with high specificity and minor cross-activity	Magnaval et al., 1991
IgG-TES-ELISA	Detection of specific total IgG in human sera, with a sensitivity of 97% and specificity of 36%	Noordin et al., 2005
IgG1-TES-ELISA	Detection of specific IgG1 subclass in human sera, with sensitivity of 60% and specificity of 76%	Watthanakulpanich et al., 2008
IgG2-TES-ELISA	Detection of specific IgG2 subclass in human sera, with sensitivity of 98% and specificity of 71%	Watthanakulpanich et al., 2008
IgG3-TES-ELISA	Detection of specific IgG3 subclass in human sera, with a sensitivity 78% and specificity of 81%	Watthanakulpanich et al., 2008
IgG4-TES-ELISA	Detection of specific IgG4 subclass in human sera, with a sensitivity of 64% and specificity of 71%	Watthanakulpanich et al., 2008
IgG4-rTES-ELISA	Detection of specific IgG4 subclass in human sera, with a sensitivity of 93% and increased specificity	Mohanmad et al., 2009
IgM/G-TES-ELISA	Detection of specific IgG or IgM in human sera, with a sensitivity of 100% and specificity of 100%	Peixoto et al., 2011
IgG-TCLA-ELISA	Detection of specific total IgG in human sera, with a sensitivity of 92% and specificity of 87%	Jin et al., 2013
IgG-dTES-ELISA	Detection of specific total IgG in human sera, with a sensitivity of 100% and specificity of 100%	Roldán et a., 2015
IgG-dTES-WB	Detection of specific total IgG in human sera, with no cross-reactivity with 32, 55 and 70 kDa of dTES	Roldán et a., 2015
IgG avidity	Measurement of IgG avidity index of humans, with a sensitivity of 44% and specificity of 83%	Boldiš et al., 2015
IgG-DiM-BSA-ELISA	Detection of specific total IgG in human sera, with a sensitivity of 92% and specificity of 95%	Elefant et al., 2016

*Footnote. IgG: immunoglobulin G; TES: *Toxocara canis* excretory/secretory antigens; ELISA: enzyme-linked immunosorbent assay; dTES: deglycosylated TES antigens; WB: western blot; IgM: immunoglobulin M; rTES: recombinant TES antigens; TCLA: antigens from *T. canis* larvae; DiM-BSA: di-O-methylated coupled bovine serum albumin.

Table 8.2. Summary of main anthelmintic drugs that have been used for the treatment of toxocariasis in humans and/or other host animals.

Drug/drug formulation*	Efficacy reported	References
ABZ	47% reduction of clinical symptoms in infected people	Stürchler et al., 1989
DEC	70% reduction of clinical symptoms in infected people	Magnaval, 1995
ABZ/PEG-LE and glucan	92% larval elimination from brains of infected mice	Hrckova et al., 2001
FBZ/PEG-LE and glucan	92% larval elimination from skeletal muscle of infected mice	Hrckova et al., 2001
ABZ/PEG-LE	79% larval elimination from brains of infected mice	Horiuchi et al., 2005
FBZ/PEG-LE	90% larval elimination from the muscles and 66% from brains of infected mice	Hrckova et al., 2007
ABZ/PEG	100% larval elimination in brain, and increased elimination from liver and lungs of infected mice	Leonardi et al., 2009
ABZ/CH	100% larval elimination from brains, and increased elimination from liver and lungs of infected mice	Barrera et al., 2010
Natural products	Some efficacy on larvae *in-vitro* and in infected mice with extracts from *Chenopodium ambrosioides* and/or Nutridesintox®	Reis et al., 2010

*Footnote. ABZ: albendazole; DEC: Diethylcarbamazine; ABZ/CH: chitosan-encapsulated ABZ; ABZ/PEG: polyethylene glycol-conjugated ABZ; ABZ/PEG-LE: liposome-encapsulated ABZ stabilized with PEG; FBZ/PEG-LE: liposome-encapsulated fenbendazole stabilized with PEG.

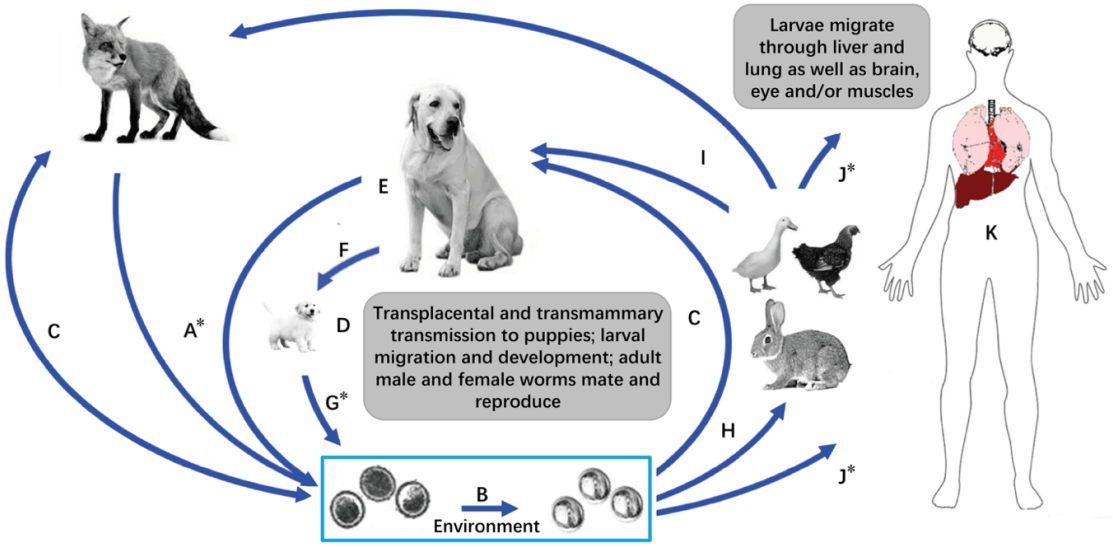


Fig. 8.1. Detailed account of the life cycle of *Toxocara canis*, presently recognised as the commonest species of *Toxocara* infecting humans. The life cycle of *Toxocara canis* involves canids (including coyotes, dogs, foxes and wolves) as definitive hosts. (A) Eggs of this parasite are released in the faeces from the canid host. (B) Eggs embryonate in the environment and become infective. (C) After ingestion by this host, infective (third-stage) larvae (L3s) hatch from the eggs, and larvae penetrate the intestinal wall. (D) In young dogs (usually < 12 weeks), larvae migrate through liver and lungs to the airways and then get swallowed and make their way to the small intestine, where fourth-stage larvae develop to adult worms (female and male), mate and reproduce. (E) In some dogs (usually > 12 weeks), oral infection can occur, but larvae tend to encyst in tissues, where they undergo arrested development. (F) In female dogs, encysted larvae are activated, usually in the last trimester of pregnancy, and undergo transplacental (~99%) transmission to pups in utero and transmammary transmission (~1%) to newborn pups. (G) Adult worms become established in the small intestine of pups, and represent a major source of egg contamination. (H) *Toxocara canis* can also be transmitted to paratenic hosts (e.g., mice, rats and rabbits) via the accidental ingestion of infective eggs; in these hosts, larvae hatch from the eggs and penetrate the intestinal wall to then migrate through/to various organs and tissues, where they encyst. (I) When a canid eats such infected paratenic hosts, adult worms develop in the small intestine and the life cycle is completed. Also (J) humans are accidental (paratenic) hosts; they can become infected by ingesting larvae in paratenic hosts or infective eggs from contaminated soil, food or water. (K) Following ingestion, such larvae are released and invade the intestinal wall to then be carried via the blood circulation to various tissues (e.g., liver, lungs, brain and muscles). Although these larvae undergo arrested development (hypobiosis) in these tissues, they often cause pathogenic effects as a result of inflammatory responses and granuloma formation associated with toxocariasis (disease). Common clinical syndromes of this disease in humans are ocular larva migrans and visceral larva migrans. The definitive diagnosis of human toxocariasis can made based on the specific identification of larvae in tissue biopsies using molecular methods; serological techniques are used for the detection of anti-*Toxocara* serum antibodies. Human toxocariasis can be prevented and/or controlled by disrupting the life cycle at key points (*); effective means include (i) treatment of pet dogs and cats (< 12 weeks of age) and stray carnivores (e.g., using baits) with an anthelmintic, and (ii) preventing humans from ingesting infective eggs from the environment or larvae in raw/uncooked tissues from infected paratenic hosts (e.g., chicken, duck and rabbit).

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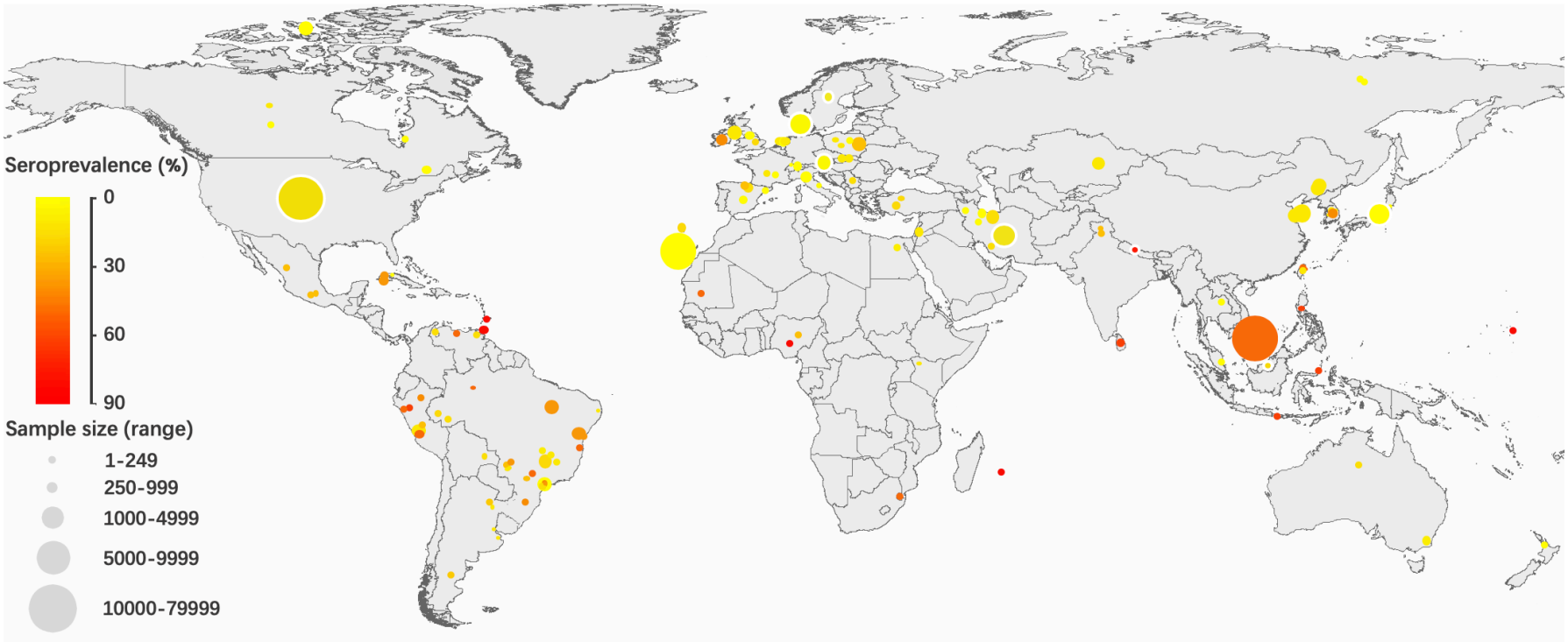


Fig. 8.2. Estimated seroprevalences and distribution of *Toxocara* infection or exposure around the world. Prevalences (%) and sample size ranges (number of individuals tested) are summarised from published works (Supplementary Table S8.1). A dot with a white halo represents a well-defined national survey or meta-analysis.

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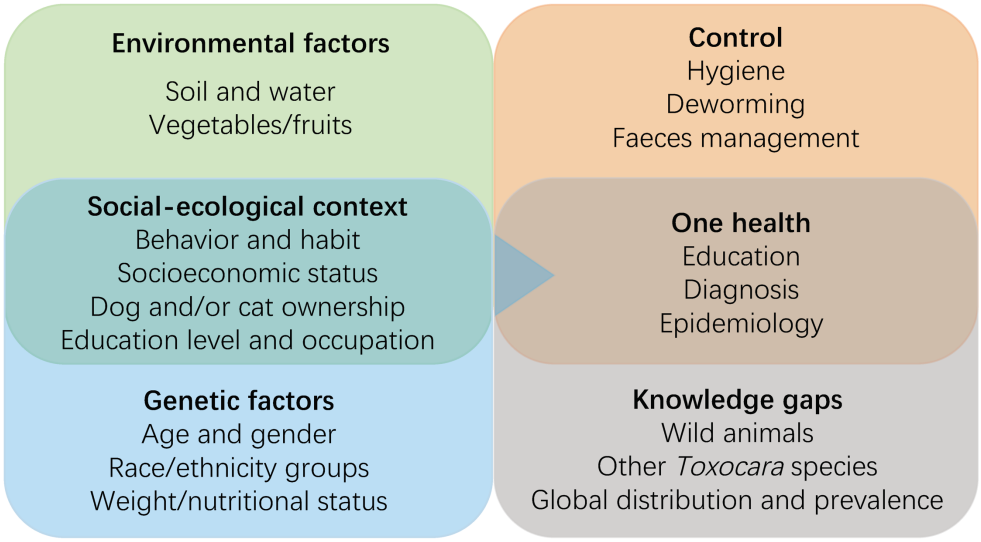


Fig. 8.3. Epidemiological determinants and risk factors of human toxocariasis. Disease manifestation of human toxocariasis can be affected by various factors, such as environment contamination with *Toxocara* eggs, human genetics (e.g., age, gender and/or physical status) as well as socio-ecological activities (dog ownership, occupation and/or education). A one-health strategy, including key components of epidemiology, diagnosis and treatment, should enhance our knowledge base and improve the control of human toxocariasis.

Supplementary Table S8.1: Published seroprevalence studies of humans for *Toxocara* infection or exposure conducted various countries and continents around the world.

Chapter 9. Comparative bioinformatic analysis suggests that specific dauer-like signalling pathway components associate with *Toxocara canis* development and migration

Abstract

Toxocara canis is quite closely related to *Ascaris suum* but its biology is more complex, involving a phase of arrested development (diapause or hypobiosis) in tissues as well as transplacental and transmammary transmission routes. In the present study, we explored and compared dauer-like signalling pathways of *Toxocara canis* and *Ascaris suum* to infer which components in these pathways might associate with this added complexity in *Toxocara canis*. Guided by information for *Caenorhabditis elegans*, we bioinformatically inferred and compared components of dauer-like signalling pathways in *Toxocara canis* and *Ascaris suum* using genomic and transcriptomic data sets. In these two ascaridoids, we also explored endogenous dafachronic acids (DAs), which are known to be critical in regulating larval developmental processes in C. elegans and other nematodes, by liquid chromatography-mass spectrometry (LC-MS). Orthologues of *Caenorhabditis elegans* dauer signalling genes were identified in *Toxocara canis* (n = 55) and *Ascaris suum* (n = 51), inferring the presence of a dauer-like signalling pathway in both species. Comparisons showed clear differences between *Caenorhabditis elegans* and these ascaridoids as well as between *Toxocara canis* and *Ascaris suum*, particularly in the transforming growth factor-β (TGF-β) and insulin-like signalling pathways. Specifically, in both *Ascaris suum* and *T. canis*, there was a paucity of genes encoding SMAD transcription factor-related protein (*daf-3*, *daf-5*, *daf-8* and *daf-14*) and insulin/insulin-like peptide (*daf-28*, *ins-4*, *ins-6* and *ins-7*) homologues, suggesting an evolution and adaptation of the signalling pathway in these parasites. In *Toxocara canis*, there were more orthologues coding for homologues of antagonist insulin-like peptides (*Tc-ins-1* and *Tc-ins-18*), an insulin receptor substrate (*Tc-ist-1*) and a serine/threonine kinase (*Tc-akt-1*) than in *Ascaris suum*, suggesting potentiated functional roles for these molecules in regulating larval diapause and reactivation. In these two ascaridoids, we detected endogenous dafachronic acids (DAs) by liquid chromatography-mass spectrometry (LC-MS), suggesting relatively conserved machinery was proposed for DA synthesis in the two ascaridoids, and endogenous Δ4- and Δ7-DAs were detected in them by LC-MS

analysis. Differential transcription analysis between *Toxocara canis* and *Ascaris suum* suggests that *ins-17* and *ins-18* homologues might regulate development and migration in *Toxocara canis* larvae in host tissues. The findings of this study provide a basis for functional explorations of insulin-like peptides, signalling hormones (i.e., DAs) and related nuclear receptors, proposed to link to development and/or parasite-host interactions in *Toxocara canis*. Elucidating the functional roles of these molecules might contribute to the discovery of novel anthelmintic targets in ascaridoids.

9.1. Introduction

Toxocara canis is an important pathogen of both animal and human health importance worldwide (see a detailed review in Chapter 9). This parasite, which is related to other ascaridoid nematodes such as *Ascaris* spp., can be directly transmitted to the human host via a faecal-oral route, and can cause toxocariasis and complications such as neurological and allergic diseases (reviewed in Chapter 9).

The biology of *Toxocara canis* is complex and involves canids (e.g., dogs, wolves and foxes) as definitive hosts, paratenic hosts such as rodents, and accidental hosts including humans (Schnieder et al., 2011; Strube et al., 2013). The eggs of this parasite are expelled in the faeces from canids, embryonate and become infective in the environment. Following the ingestion of infective eggs by the canid host, infective, third-stage larvae (L3s) emerge from the eggs, penetrate the intestinal wall and migrate to the liver and lungs (hepato-pulmonary migration). In young dogs (< 12 weeks), larvae migrate to the airways and then get swallowed and make their way to the small intestine, where fourth-stage larvae (L4s) develop to adult worms (female and male), mate and reproduce. In dogs of ≥ 12 weeks, oral infection can occur, but larvae tend to encyst in various tissues (including muscles, brain and nerves), where they undergo hypobiosis (i.e., arrested development or diapause). In female dogs, encysted larvae become activated (in the last trimester of pregnancy), most of which undergo transplacental transmission to the foetuses in utero (~99%), and a minority of which (~1%) undergo transmammary (transcolostoral) transmission to newborn pups (Burke and Roberson, 1985). Adult worms eventually establish in the small intestine of pups, and reproductively-active female worms are a major source of egg contamination. *Toxocara canis* can also be transmitted to accidental (paratenic) hosts (e.g., rats, mice and rabbits) via accidental ingestion of infective eggs; in such hosts, larvae hatch from the eggs, penetrate the intestinal wall and then migrate to various organs and tissues, where they cause disease and/or encyst. If a canid eats infected tissues (containing larvae) from such a paratenic host, adult worms can develop in the small intestine (Schnieder et al., 2011; Strube et al., 2013).

Humans are accidental hosts. They can become infected by ingesting infective eggs from contaminated soil, food or water, or larvae in tissues from infected paratenic hosts

(reviewed in Chapter 9). Following ingestion, infective larvae are released and invade the intestinal wall, and are then carried via the blood circulation to various tissues (including liver, lungs, muscles, central nervous system). Although these larvae undergo arrested development in these tissues, during prior migration they frequently cause pathogenic effects due to associated mechanical damage, inflammatory responses and granuloma formation (Maizels, 2013), leading to the disease toxocariasis, of which there are four main clinical forms (visceral larva migrans, ocular larva migrans, neurotoxocariasis and covert toxocariasis) (reviewed in Chapter 9). Such larvae in tissues have also been implicated in neurodegenerative disorders (e.g., epilepsy, idiopathic Parkinson's disease and dementia) and in allergic diseases (e.g., asthma and pruritus) (Gavignet et al., 2008; Çelik et al., 2013; Fan et al., 2015; Aghaei et al., 2018; Fialho et al., 2018).

Interestingly, *Toxocara canis* has a marked tropism for the central nervous system (cerebrum) (Janecek et al., 2014). Larvae that migrate through the brain downregulate lipid/cholesterol biosynthesis (Janecek et al., 2015). There is evidence that prolactin (pituitary hormone) plays a role in activating arrested larvae of *Toxocara canis* (Jin et al., 2008), and some studies have implicated this hormone in regulating larval growth and motility in *Toxocara canis*, infection intensity and host immune responses (Chávez-Güitrón et al., 2016; Del Río-Araiza et al., 2018). The information from these studies indicates that lipid or hormone signalling plays critical roles in the migration, diapause and host interplay of *Toxocara canis* larvae. Roles of similar signalling pathways in regulating developmental processes have been described for the free-living nematode *Caenorhabditis elegans* (see Motola et al., 2006; Bethke et al., 2009; Hochbaum et al., 2011). Specifically, steroid hormone signalling, particularly the dafachronic acid-DAF-12 module, has been recognised as a 'check-point' for diapause (dauer) in this worm (Hu, 2007; Fielenbach and Antebi, 2008; Ogawa et al., 2009; Wang et al., 2009), and has been proposed to relate to an endocrine mechanism which appears to be conserved between free-living and parasitic nematodes (Hotez et al., 1993; Bento et al., 2010; Lee and Schroeder, 2012; Crook, 2014). However, little is known about the signalling pathways in parasitic nematodes and the differences in such pathways between *Toxocara* and other ascaridoids such as *Ascaris suum* (see Gilabert et al., 2016), which, unlike *Toxocara canis*, does not undergo transplacental or transmammary transmission (Crompton, 2001; Holland, 2013). Profound knowledge of dauer and associated signalling pathways in

Caenorhabditis elegans enables comparative studies in parasitic nematodes utilising transcriptomic and genomic data sets and tools (Gasser, 2013; Gasser et al., 2016), in the absence of functional genomic data. Here, guided by information and data for *Caenorhabditis elegans*, we inferred dauer-like signalling pathways of *Toxocara canis* and *Ascaris suum*, and identified unique components that we hypothesise regulate the development and/or other molecular processes in *Toxocara canis* in the mammalian host.

9.2. Methods

9.2.1. Draft genomes and transcriptomes

Genome assemblies and annotations for *Caenorhabditis elegans* (BioProject PRJNA13758), *Toxocara canis* (BioProjects PRJEB533 and PRJNA248777) and *Ascaris suum* (BioProjects PRJNA80881 and PRJNA62057) were obtained from WormBase (Lee et al., 2018) and ParaSite at WormBase (Howe et al., 2016). Transcriptomic data sets of *Toxocara canis* and *Ascaris suum* (BioProjects PRJNA248777 and PRJNA80881) were taken from NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) (Jex et al., 2011; Zhu et al., 2015; Wang et al., 2017; Zhou et al., 2017). New versions of the transcriptomes of *Toxocara canis* and *Ascaris suum* were assembled de novo using the program Trinity v.2.4.0 (Grabherr et al., 2011; Hass et al., 2013).

9.2.2. Identification of dauer signalling gene homologues

Genes (n = 107) representing the canonical dauer signalling pathway [i.e., cyclic guanosine monophosphate (cGMP), transforming growth factor-β (TGF-β), insulin/insulin-like growth factor 1 (IGF-1), and steroid hormone signalling pathways] in *Caenorhabditis elegans* were available from published information (Hu, 2007; Stoltzfus et al., 2012; Gilabert et al., 2016). Protein sequences (n = 182) and functional information were obtained from WormBase (WS261) (https://www.wormbase.org). Homologues were predicted by exhaustive homology searching of *Caenorhabditis elegans* protein sequences (using BLAT v.35 and tblastn v.2.5.1) against the genome and transcriptome assemblies of *Toxocara canis* and *Ascaris suum*. In addition, Pfam, PANTHER and

SUPERFAMILY conserved domain architectures (using InterProScan v.5.15.54) (Zdobnov and Apweiler, 2001; Jones et al., 2014) of the dauer signalling gene products were used to predict homologues from the original gene predictions and de novo-assembled transcripts of these two ascaridoid species, using an established approach (Stroehlein et al., 2018). Predicted transcripts of potential homologues were matched (using blastx v.2.5.1, *e*-value: $\leq 10^{-5}$) to proteins of *Caenorhabditis elegans* (BioProject PRJNA13758.WS261) to verify their identity.

9.2.3. Curation of genes and classification of orthologues

Homologous sequences were manually curated using a recently described workflow (Stroehlein et al., 2018). In brief, identified gene and transcript sequences were mapped to the genome assemblies of each *Toxocara canis* and *Ascaris suum* using the program BLAT v.35 (Kent, 2002). Transcripts that mapped to the same coding region were re-assembled using the program CAP3 for possible extensions (Huang and Madan, 1999). Full-length transcripts were used to refine the corresponding gene models using the program Exonerate v.2.2.0 (Slater and Birney, 2005). Gene products were predicted from the curated coding DNA sequences (CDSs) using ORFfinder (Rombel et al., 2002). Gene orthologues were classified according to groups inferred by OrthoMCL (*e*-value: $\leq 10^{-5}$; sequence similarity: $\geq 30\%$) (Li et al., 2003).

9.2.4. Comparative analyses

Sequence similarities of the classified orthologues were compared by pairwise alignment using EMBOSS Needle (the Needleman-Wunsch algorithm) (Li et al., 2015). Domain architectures of inferred proteins were assigned using InterProScan v.5.15.54. Specifically, homologues of genes encoding insulin-like peptides were identified based on their sequence domain signatures. The inferred protein sequences were compared with the insulin/insulin-like peptides of *Caenorhabditis elegans* using blastp (*e*-value $\leq 10^{-5}$) to infer their identity. For inferred insulin-like peptides, conserved patterns and motifs were searched using the programs Pratt v.2.1 (Jonassen et al., 1995) and MEME v.5.0.2

(Bailey et al., 2015). The relationships of insulin-like peptides were verified manually using OrthoMCL (*e*-value: ≤ 10^{-5}; sequence similarity: ≥ 50%).

9.2.5. Transcriptional analysis

Available RNA-seq reads from egg, and first- (L1), second- (L2), third- (L3, recovered from eggs, liver and lungs) and fourth-stage larvae and/or adults of *Toxocara canis* and of *Ascaris suum* (see Jex et al., 2011; Zhu et al., 2015; Zhou et al., 2017) were used for transcriptional analyses and comparisons. In brief, paired-end reads were mapped to individual curated CDSs using Bowtie2 v.2.1.0 within the software package RSEM v.1.2.11 (Li and Dewey, 2011; Langmead and Salzberg, 2012). Levels of messenger RNA (mRNA) transcription were recorded in transcripts per million (TPM). For individual developmental stages, transcription profiles for individual orthologues were displayed in a heat-map using the program heatmap.2 (in R v.3.5.1).

9.2.6. LC-MS/MS

For each *Toxocara canis* and *Ascaris suum*, lipids were extracted from four individual male and four individual female adults using an established lipid extraction method (Folch et al., 1957; see Chapter 4). In brief, individual samples (1 mg dry weight; 4 replicates) were suspended in ice cold 40% methanol and homogenised using zirconium oxide beads (ZROB05, Next Advance, USA). A chloroform:methanol (2:1) mix was used to separate the aqueous and organic phases by centrifugation at 10,000× *g* for 10 min at room temperature (24 °C). The organic phase was retained, dried and resuspended in methanol for subsequent mass spectrometric analysis using an Orbitrap Fusion Lumos mass spectrometer coupled to an Ultimate 3000 UHPLC (Thermo Fisher Scientific, San Jose, CA, USA). Commercially available dafachronic acids (25S)-Δ7-DA and (25S)-Δ4-DA (exact mass: 413.3061) (cat no. 23017-97-2; Cayman Chemical Company) were used as reference standards for the identification of endogenous DAs.

9.3. Results

9.3.1. Dauer signalling orthologues

Based on the information available for *Caenorhabditis elegans*, we identified 55 and 51 orthologues encoding signalling molecules in *Toxocara canis* and *Ascaris suum*, respectively (Supplementary Tables S9.1-9.4). These numbers are markedly lower than for *Caenorhabditis elegans* (n = 107) and relate mainly to less TGF-β and insulin/insulin-like signalling components in the ascaridoids. Specifically, orthologues inferred to represent SMAD transcription factors (*daf-3, daf-8* and *daf-14*), SKI family transcriptional co-repressor (*daf-5*), insulin (*daf-28*), insulin-like peptides (e.g., *ins-4*, *ins-6* and *ins-7*), serine/threonine-protein kinase (*akt-2*), bZip transcription factor (*skn-1*), 14-3-3 protein (*par-5*) and iron/manganese superoxide dismutase (*sod-3*) were not detected in either *Toxocara canis* or *Ascaris suum*, whereas two orthologues encoding heat-shock protein 90 (*daf-21*) were identified in both species (Fig. 9.1). A comparison indicated more orthologues coding for a cGMP-dependent protein kinase, insulin-like peptides, an insulin receptor substrate and a serine/threonine-protein kinase (*egl-4*, *ins-1*, *ins-18*, *ist-1* and *akt-1*) in *Toxocara canis* compared with *Ascaris suum* (see Fig. 9.1). More transcript isoforms were predicted for *Toxocara canis* orthologues, such as *Tc-akt-1* (n = 5) and *Tc-daf-12* (n = 18), than for *Ascaris suum* (see Supplementary Tables S9.3 and S9.4).

9.3.2. Insulin-like peptide-coding genes and their relationships

We identified 10 and 5 sequences encoding signatures characteristic of the insulin-like superfamily in *Toxocara canis* and *Ascaris suum*, respectively (Supplementary Table S9.5). Although marked sequence diversity (23-77%) was seen among the inferred insulin-like peptides of *Caenorhabditis elegans*, *Toxocara canis* and *Ascaris suum* (Supplementary Table S9.5), two characteristic motifs (RLCGRKLIKAVQSLC and CCSKGCTDEDJKKYC; P-value: $\leq 10^{-5}$) and one conserved sequence pattern (C-C; fitness = 8.34) were discovered (Fig. 9.2). Specifically, the proteins inferred for *Toxocara canis* and *Ascaris suum* had significant sequence similarity (blastp e-value cut-off: $\leq 10^{-5}$) to *Ce*-INS-1, *Ce*-INS-12, *Ce*-INS-17, *Ce*-INS-18 or *Ce*-INS-32 (Supplementary Table

S9.5). Relationships among the insulin-like peptides for these species were supported by their orthologous groups (n = 12; e-value: ≤ 10^{-5}; sequence similarity: ≥ 50%) (Supplementary Table S9.5). Apart from the orthologues inferred (i.e., INS-1, INS-17 and INS-18), one more homologue of *Ce-ins-1* was inferred for *Toxocara canis*, and novel insulin-like peptides were predicted for *Toxocara canis* (n = 4) and *Ascaris suum* (n = 2) (Fig. 9.2; Supplementary Table S9.5).

9.3.3. Dafachronic acid biosynthesis machinery

We inferred 6 orthologues to be involved in the biosynthesis of DAs. Specifically, the identification of orthologues *ncr-1*, *daf-36*, *dhs-16*, *strm-1*, *emb-8* and *daf-9* indicated a relatively conserved biosynthetic pathway for DA (i.e., Δ7) among *Caenorhabditis elegans*, *Toxocara canis* and *Ascaris suum* (Fig. 9.3A), although the orthologue of *Ce-hsd-1* was not identified in either *Toxocara canis* or *Ascaris suum*.

Endogenous DAs (Δ4 and Δ7) were detected by LC-MS in the female and male adults of *Toxocara canis* and *Ascaris suum*, with mass errors estimated at 0.5 and 0.4 parts per million (ppm), respectively (Fig. 9.3B). Specifically, Δ7-DA was dominant in *Toxocara canis*, whereas Δ4-DA was in *Ascaris suum* (Fig. 9.3). For both species, the relative abundance of DAs in female adults was higher than in male adults (P > 0.05) (Fig. 9.3C).

9.3.4. Developmental transcription of dauer signalling components

We compared levels of mRNA transcription of individual dauer signalling orthologues in L3 and adult (female and male) stages of *Toxocara canis* (Supplementary Table S9.6). For some orthologues, including *Tc-gpa-3*, *Tc-daf-11* and *Tc-tax-4* (cGMP signalling), *Tc-asna-1* and *Tc-ins-18* (insulin-like signalling), and *Tc-daf-9* (steroid hormone signalling), transcription was higher in the L3 than the adult stage (Supplementary Table S9.6). We also compared mRNA transcription levels of individual dauer signalling orthologues in seven distinct developmental stages of *Ascaris suum* (Supplementary Table S9.6). Although similar transcriptional profiles were observed in egg and L1 stages, marked variation was seen among L2, L3 (recovered from eggs, liver and lungs) and L4 stages of *Ascaris suum*, particularly for the genes *As-gpa-3*, *As-daf-11* and *As-tax-4*

(cGMP signalling), *As-asna-1* and *As-daf-2* (insulin-like signalling), and *As-daf-9* (steroid hormone signalling) (Fig. 9.4). A comparative analysis showed high levels of transcription for the orthologues *daf-21* and *ftt-2* in the stages of *Toxocara canis* and *Ascaris suum* studied, and low levels for *ins-17* and *ins-18* in the latter species (Supplementary Table S9.6).

9.4. Discussion

Based on current genomic and transcriptomic datasets, we identified orthologues of the dauer signalling genes in *Toxocara canis* and compared them with those of *Ascaris suum* using a bioinformatic strategy for gene curation and classification. We compared these components, proposed the biosynthetic pathways of DA and investigated developmental transcription of identified orthologues.

The identification of key dauer-associated signalling gene orthologues implies dauer-like signalling pathways in *Toxocara canis* and *Ascaris suum*. Although dauer signalling gene homologues had been identified previously in a range of parasitic nematodes, including *Toxocara canis* and *Ascaris suum* (see Gilabert et al., 2016), orthologues were defined here using an effective approach for curation and classification (Stroehlein et al., 2018). Specifically, the sequence re-assembly strategy used increased the likelihood of identification. For instance, we identified orthologues of *Ce-ins-1* and *Ce-dhs-16* in both *Toxocara canis* and *Ascaris suum*, which were not reported previously (Gilabert et al., 2016). In addition, the present sequence-based classification strategy improved the accuracy of annotation. Although *Ce-bra-1* homologues had been identified (Gilabert et al., 2016), the orthologue of this gene was not identified herein in *Toxocara canis* or *Ascaris suum*. Specifically, orthologues of *Ce-ftt-2* and *Ce-par-5* (encoding 14-3-3 proteins) were classified based on reciprocal BLAST searches and OrthoMCL grouping. Thus, the accurate identification and classification of orthologues provided a basis for reliable comparative studies.

The present findings indicated a divergent evolution and adaptation of the dauer-like signalling pathway among the three nematodes studied. Compared with *Caenorhabditis elegans*, the reduced number of genes coding for SMAD-related transcription factors and insulin/insulin-like peptides in *Toxocara canis* and *Ascaris suum* suggest divergences in

both TGF-β and insulin-like signalling between the free-living and the two parasitic nematodes. Specifically, orthologues of *Ce-daf-3*, *Ce-daf-5*, *Ce-daf-8* and *Ce-daf-14* were not detected in *Toxocara canis* or *Ascaris suum*, suggesting a uniqueness in TGF-β signalling in these two species (clade III), as suggested previously by other workers for other parasitic nematodes such as *Trichinella spiralis* and *Trichuris suis* (clade I) (see Beall and Pearce, 2002; Gilabert et al., 2016). In addition, apart from *ins-1*, *ins-17* and *ins-18*, no orthologue of any of the other insulin/insulin-like peptide-coding genes (*n* = 37) of *Caenorhabditis elegans* (clade V) was found, implying a contraction of the gene family representing these signalling molecules in the two ascaridoids (clade III) studied here. Reduced numbers of genes encoding insulin-like peptides have been reported also in other parasitic nematodes, including *Strongyloides stercoralis* (clade IV) (see Stoltzfus et al., 2012).

Distinctiveness in signalling between *Toxocara canis* and *Ascaris suum* might relate to differences in biology and/or developmental regulation. Although the dauer-like signalling pathways of *Toxocara canis* and *Ascaris suum* were similar, the increased number of orthologues in the former species indicates a distinction in TGF-β and insulin-like signalling (Fig. 9.1D) which might associate with entry into and exit from arrested larval development in *Toxocara canis*. First, the three *Tc-ins-1* and two *Tc-ins-18* paralogues encode antagonist insulin-like peptides, which are recognised to function in promoting dauer formation in *Caenorhabditis elegans* (see Pierce et al., 2001; Matsunaga et al., 2012). The novel insulin-like peptides (*n* = 4; *Tc-ins-1n* to *Tc-ins-4n; n = novel*) predicted for *Toxocara canis* might also be involved in regulating larval development, but this proposal needs to be assessed. Secondly, the two *Tc-ist-1* paralogues likely encode insulin receptor substrates, which function together with phosphoinositide 3-kinase (PI3K) adaptor/regulatory subunit to potentiate dauer-associated *daf-2*/insulin-like signalling, as known for *Caenorhabditis elegans* (see Wolkow et al., 2002). Thirdly, the two *Tc-akt-1* orthologues encode serine/threonine kinases Akt/protein kinase B, which play a role in antagonising the fork head transcription factor DAF-16 when activated by phospholipid products from PI3K (Paradis and Ruvkun, 1998). Therefore, compared with *Ascaris suum*, it appears that *Toxocara canis* produces more antagonist molecules (INS-1 and INS-18) which may trigger a stronger inhibitive effect on the fork head transcription factor DAF-16 (via phosphorylation of a serine/threonine kinase) to enable arrested

development, particularly when migrating through and/or encysting in host tissues. In addition, the relatively high numbers of transcript isoforms for *Tc-akt-1* (*n* = 5) and *Tc-daf-12* (*n* = 18) might indicate rapid adaptive expression and/or plastic functional roles, similar to those of *daf-16* reported for *Caenorhabditis elegans* (see Kwon et al., 2010). However, although two *Tc-egl-4* paralogues were identified in *Toxocara canis*, their functions are unclear, because orthologues coding for SMAD-related transcription factors (Daniels et al., 2000) were not identified in this nematode. Clearly, detailed functional explorations of these molecules need to be undertaken to explain their roles in the development, migration and/or hypobiosis of *Toxocara canis* larvae. Significant progress has been made through the development of a functional genomics platform for *Ascaris suum* (see McCoy et al., 2015), which suggests that a similar system might be established for *Toxocara canis*, in order to explore the functional roles of signalling molecules in this species.

To investigate dauer-like signalling pathway components in *Toxocara canis* and *Ascaris suum*, we also analysed transcription profiles across developmental stages, employing RNA-sequence data for pooled worms. The consistently high messenger RNA levels of *daf-21* and *ftt-2* suggest key roles for these genes and their products in larval development, supported, to some extent, by information for *Caenorhabditis elegans* (see Hu, 2007). By contrast, variable transcription levels for the genes *gpa-3*, *daf-11* and *tax-4* (cGMP signalling), *asna-1* (insulin-like signalling) and *daf-9* (steroid hormone signalling) among developmental stages, particularly L3 stages in eggs, liver and lung, might suggest plastic, but crucial roles in regulating developmental processes and/or host-parasite interactions. In addition, differential transcriptional levels of the genes *ins-17* and *ins-18* in the L3 stage between *Toxocara canis* and *Ascaris suum* suggest a distinctive signalling mechanism in the host animal, since *Ce-ins-17* and *Ce-ins-18* promote dauer formation in *Caenorhabditis elegans* (see Matsunaga et al., 2012a, 2012b). This difference might suggest unique roles for selected insulin-like peptides in regulating arrested development in host tissues or parasite-host interactions (Beall and Pearce, 2002; Lok, 2016). This hypothesis warrants testing through large-scale, integrative ‘omic investigations.

Comparisons with *Caenorhabditis elegans* implied relative conservation in the biosynthetic machinery for DA between *Toxocara canis* and *Ascaris suum*. The

orthologues of *Ce-ncr-1*, *Ce-daf-36*, *Ce-dhs-16*, *Ce-emb-8* and *Ce-daf-9* likely function in the trafficking, catalysation and modification of cholesterol destined for the biosynthesis of DAs, known to be critical for regulating larval development in *Caenorhabditis elegans* and *Strongyloides stercoralis* (see Motola et al., 2006; Gerisch et al., 2007; Albarqi et al., 2016; Butcher, 2017). The biosynthetic machinery proposed for ascaridoids is supported by the identification of endogenous Δ4- and Δ7-DAs in the adult stage of *Toxocara canis* and *Ascaris suum*, although only Δ7-DA was detected in the larval stage of *Haemonchus contortus* (see Chapter 7). Interestingly, there are differential abundance of Δ4- and Δ7-DAs in the adult stage of *Toxocara canis* and *Ascaris suum*, suggesting functional distinctions in specific biological processes between these species. Based on the information for *Caenorhabditis elegans* and some parasitic nematodes (Hannich et al., 2009; Stoltzfus et al., 2012), the endogenous biosynthesis of Δ7-DA might be regulated by the *strm-1* gene in *Ascaris suum*. Although similar components of the DA synthesis were predicted for both *Toxocara canis* and *Ascaris suum*, the functionality of this machinery in these species needs to be shown. By contrast, an orthologue of *Ce-hsd-1*, which likely functions in the biosynthesis of other DAs (Patel et al., 2008), was not detected in either *Toxocara canis* or *Ascaris suum*, suggesting that both of these ascaridoid nematodes have a simplified machinery to synthesise these signalling hormones. Although it seems that there is a relatively conserved steroid hormone signalling module in these ascaridoids, future work should focus on verifying the biosynthesis signalling module and the functional roles of DAs in these worms. Specifically, although it has been reported that prolactin plays a role in re-activating arrested larvae of *Toxocara canis* (see Jin et al., 2008), the proposed role for DAs ("bile acid-like steroids") in larval reactivation (relating particularly to ensuing transplacental and/or transmammary transmission) and their associated signalling pathway(s) remain to be explored (Tissenbaum et al., 2000; Ogawa et al., 2009; Albarqi et al., 2016). A better understanding of these areas, particularly signalling hormones and their nuclear receptors, would provide insight into regulatory processes in larval development and might enable the discovery of new anthelmintic targets (Wang et al., 2009; Wang et al., 2017; Patton et al., 2018).

In conclusion, the present study reveals distinctiveness in the TGF-β and insulin-like signalling pathways among *Caenorhabditis elegans*, *Toxocara canis* and *Ascairs suum*,

but indicates similarity in the steroid hormone signalling pathway between the two ascaridoid nematodes. Inferring the elements of the dauer-like signalling pathway in these ascaridoids provides a basis for future explorations of the functional roles of these elements in each species using a reliable functional genomics platform. Understanding these processes could enlighten developmental processes and host-parasite interactions for these ascaridoids and facilitate the discovery of novel intervention strategies against the diseases that these parasites cause.

9.5. References

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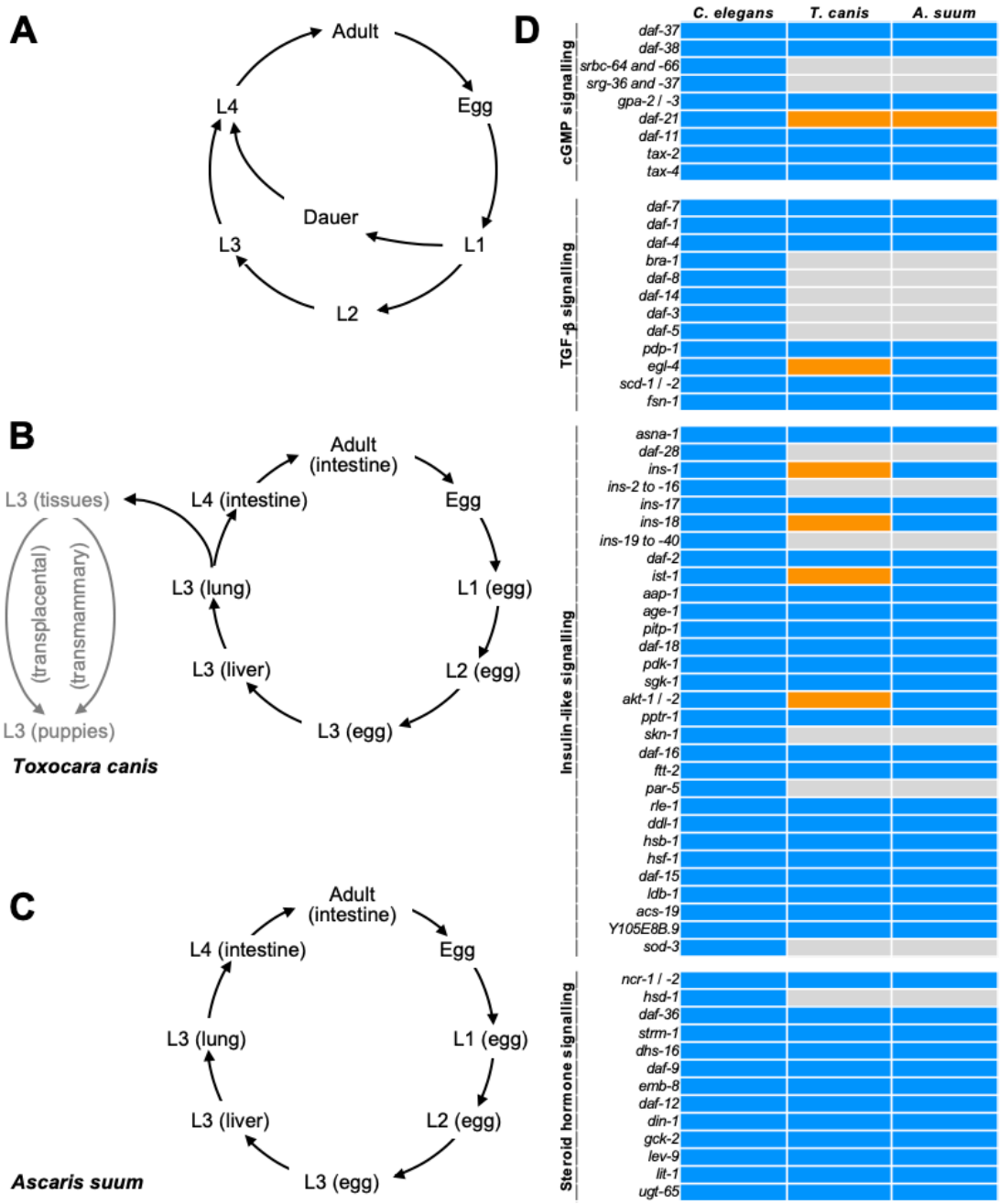
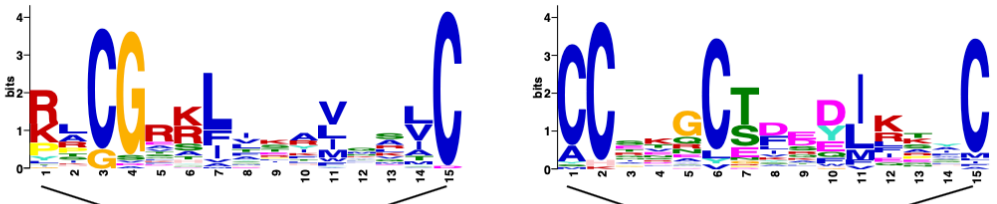


Fig. 9.1. Life-cycle and dauer signalling orthologues of *Caenorhabditis elegans*, *Toxocara canis* and *Ascaris suum*. Schematic representation of the life-cycles of *Caenorhabditis elegans* (A), *Toxocara canis* (B) and *Ascais suum* (C). Specifically, the third-stage larvae (L3s) of *Toxocara canis* can arrest in the tissues for years, and can be reactivated in the female dog (bitch) in the last trimester of pregnancy and then migrate to uterus or mammary glands (postpartum), leading to transplacental or transmammary transmission to offspring (grey arrows). (D) Dauer signalling gene orthologues in *Toxocara canis* and *Ascaris suum* are indicated (in blue) and compared to *Caenorhabditis elegans* genes. Increased numbers of orthologues are identified in *Toxocara canis* and/or *Ascaris suum* (orange). Orthologues not inferred for *Toxocara canis* and/or *Ascaris suum* are indicated in grey.



***Ce*-DAF-28**	RAVRVPGVAV	RACGRRLVPYVWSVC	GDACEPQEGI	.....	PQEGIDIATQ	CCTYQCTAEYIQTAC	CPRLLL
***Ce*-INS-1**	SSPTPSDASI	RLCGSRLTTTLLAVC	RNQLCTGLTA	.....	QQKRGGIATE	CCEKRCSFAYLKTFC	CNQDDN
***Ce*-INS-11**	QGAPHHDKRH	TACVLKIFKALNVMC	NHEGDADVLR	.....	ADVLRRTASD	CCRESCSLTEMLASC	TLTSSEESTR
***Ce*-INS-12**	SAAPSHEKTH	KKCSDKLYLAMKSLC	SYRGYSEFLR	.....	SEFLRNSATK	CCQDNCEISEMMALC	VVAPNFDDDL
***Ce*-INS-13**	KMCQYSKKKY	KICGVRALKHMKVYC	TRGMTRDYGK	.....	TRDYGKLLVT	CCSKGCNAIDIQRIC	L
***Ce*-INS-14**	FINFAVSSED	IKCDAKFISRITKLC	IHGITEDKLV	.....	EDKLVRLLTR	CCTSHCSKAHLKMFC	TLKPHEEEPH
***Ce*-INS-15**	QPRDNKHHSY	RSCGESLSRRVAFLC	NGGAIQTEIL	.....	IQTEILRALD	CCSTGCTDKQIFSWC	DFRKLTRKEK
***Ce*-INS-16**	FAPEIHGREL	KRCSVKLFDILSVIC	GTESDAEILQ	.....	AEILQKVAVK	CCQEQCGFEEMCQHA	NLKIDKI
***Ce*-INS-17**	RAKHGSLKLC	PPGGASFLDAFNLIC	PMRRRRRSVS	.....	GSLLGRTMNM	CCETGCEFTDIFAIC	NPFG
***Ce*-INS-18**	QQADGRMKMC	PPGGSTFTMAWSMSC	SMRRRKRDVG	.....	APSIRQLQTI	CCQVGCNVEDLLAYC	API
***Ce*-INS-19**	YEVLMLFGYK	RTCGRRLMNRINRVC	VKDIDPADID	.....	IDPKIKLSEH	CCIKGCTDGWIKKHI	CSEEVLNFGF
***Ce*-INS-2**	TPNRASRVQK	RLCGRRLILFMLATC	GECDTDSSED	.....	TDSSEDLSHI	CCIKQCDVQDIIRVC	CPNSFRK
***Ce*-INS-20**	KHHHHHHRHK	GYCGVKAVKKLKQIC	PDLCSNVDDN	.....	SNVDDNLLME	MCSKNLTDDDILQRC	CPE
***Ce*-INS-23**	TDAHSELHVR	RVCGTAIIKNIMRLC	PGVPACENGE	.....	ENGEVPSPTE	YCSMGYSDSQVKYLC	CPTSQ
***Ce*-INS-24**	GLIRANQGPQ	KACGRSMMMKVQKLC	AGGCTIQNDD	.....	TIQNDDLTIK	SCSTGYTDAGFISAC	CPSGFVF
***Ce*-INS-25**	QLSEAKPEAQ	RRCGRYLIRFLGELC	NGPCSGVSSV	.....	GVSSVDIATI	ACATAVPIEDLKNMC	CPNL
***Ce*-INS-26**	GNHHHGTKAG	LTCGMNIIERVDKLC	NGQCTRNYDA	.....			
***Ce*-INS-27**	AFLAPSTAAK	RRCGRRLIPYVYSIC	GGPCENGDII	.....			
***Ce*-INS-28**	LLAIVTSTAS	PTCGRALLHRIQSVC	GLCTIDAHHE	.....	IDAHHELIAI	ACSRGLGDKEIIEMC	CPI
***Ce*-INS-29**	SVATADFGAQ	RRCGRHLVNFLEGLC	GGPCSEAPTV	.....	EAPTVELASW	ACSSAVSIQDLEKLC	CPSNLA
***Ce*-INS-3**	MARSRRGDKV	KICGTKVLKMVMVMC	GGECSSTNEN	.....	SSTNENIATE	CCEKMCTMEDITTKC	CPSR
***Ce*-INS-30**	EPVVAAQGAK	KTCGRSLLIKIQQLC	HGICTVHADD	.....	TVHADDLHET	ACMKGLTDSQLINSC	CPPIPQTPFV
***Ce*-INS-31**	FFGPSFKALE	RSCGPKLFTRVKTVC	GEDINVDNKV	.....	YKIEWELMDN	CCEVVCEDQWIKETF	CRAPRFNFFG
***Ce*-INS-32**	LAVKSRSRRE	LICGRRLSKTVTNLC	VEMNPQKEED	.....	QKEEDIATKC	CKNKGCSREYIKSIM	CPDE
***Ce*-INS-33**	RHRRHRHHGQ	KHCGTKIVRKLQMLC	PKMCTISDDT	.....			
***Ce*-INS-37**	PVPNAAFLPY	RSCGSHLVHRAFEAC	SGKKDRSSDV	.....	RSSDVDLWKM	CCKDECTDLDIKESL	CKYASQGYGV
***Ce*-INS-38**	SQRSHVFSYK	KHCGRRIVSLVQACD	RIDHDLSIDC	.....	DRIDHDLSID	CCTQNCSSEFVKKIM	CPSKL
***Ce*-INS-4**	RARRVPAGEV	RACGRRLLLFVWSTC	GEPCTPQEDM	.....	PQEDMDIATV	CCTTQCTPSYIKQAC	CPEK
***Ce*-INS-5**	ISRADRHTNY	RSCALRLIPHVWSVC	GDACQPQNGI	.....	PQNGIDVAQK	CCSTDCSSDYIKEIC	CPFD
***Ce*-INS-6**	ARRVPAPGET	RACGRKLISLVMAVC	GDLCNPQEGK	.....	PQEGKDIATE	CCGNQCSDDYIRSAC	CP
***Ce*-INS-7a**	TRRVPDEKKI	YRCGRRIHSYVFAVC	GKACESNTEV	.....	SNTEVNIASK	CCREECTDDFIRKQC	CP
***Ce*-INS-7b**	TRRVPDEKKI	YRCGRRIHSYVFAVC	GKACESNTEV	.....	SNTEVNIASK	CCREECTDDFIRKQC	CP
***Ce*-INS-8**	RVRRVPEQKN	KLCGKQVLSYVMALC	EKACDSNTKV	.....	SNTKVDIATK	CCRDACSDEFIRHQC	CP
***Ce*-INS-9**	ARRTLETEKI	YRCGRKLYTDVLSAC	NGPCEPGTEQ	.....	PGTEQDLSKL	CCGNQCTFVEIRKAC	CADKL
***Tc*-INS-1-1**	SDRSDATGGI	RLCGQKLTRTLMAIC	RNELCGGYFS	.....	PTKRDGVATE	CCVHRCTYRYLKTFC	CDGEMWNE
***Tc*-INS-1-2**	VIVIASTTEL	RLCGKKLTFVLGRLC	NHRYNAPTKE	.....	HYTRHGIAHD	CCTNRCSYQYLKTYC	APDNAFEQFR
***Tc*-INS-1-3**	SRARKRSPAS	RLCGNKLVEAIVKMC	NGCVKPAGGK	.....	VVKRSTLTEK	CCVHRCTFEDMKSFC	CE
***Tc*-INS-17**	KPKFGTLKLC	PPGGRSFFEAFQLAC	PMRRKRDISL	.....	PATIDEMMKI	CCIRGCEFSDFFPYC	GPFGGWL
***Tc*-INS-18-1**	DERFGSLILC	PAGGQAFATAYMMAC	DMRRRKRDAI	.....	PATLTEMQNI	CCAVGCELRDLFSYC	DPFGGFNS
***Tc*-INS-18-2**	APNVGVLKMC	PPGGETFAIAWQLTC	GMRKKRQAPS	.....	PLSLTEMMHY	CCRFGCTFRDLLPYC	DPFGGWDS
***Tc*-INS-novel1**	LASSRGTNAH	RLCGFKLAKIIRQVC	EVAECNWDDE	.....	DERVISEKCC	AHPSGCTIDYIEKWC	CRNEHKYDTV
***Tc*-INS-novel2**	IKPSKGSELI	KACGDRLETIMNSIC	SYREQKLPCY	.....	SAQSAVIVSK	CCNVGCRKNEIENVC	CFTEKCLQNC
***Tc*-INS-novel3**	LCASQSSAKR	KLCGAALQSHLVRVC	TFAYERTPCF	.....	ARAFTGIANQ	CCEEGCTVLDMRATC	CFKLSCLKRC
***Tc*-INS-novel4**	MSALCRGMGL	RLCGEDLQEAMIRIC	TFADQKSPCF	.....	RLREGSLMSR	CCEKNCTLDDLRTAC	CFSLRCLQRC
***As*-INS-1**	SGAMWNNDYG	RLCGRRLTILVTKLC	AASGCMANLL	.....	DLQEESISKQ	CCAIGCSFEQIRTFC	CEIDKHDKNS
***As*-INS-17**	KPKFGTLKLC	PPGGRSFFEAFQLAC	PMRRKRDISL	.....	PATLPEIMKI	CCLRGCEFSDFFPHC	GPFSVWH
***As*-INS-18**	VPVAGVLKMC	PPGGETFATAWQLTC	GMRKKRETSE	.....	PLSLTEMMHY	CCQFGCTFRDLLPYC	DPFGGWDS
***As*-INS-novel1**	VISSSGGDAH	RLCGFKLAKIIAQVC	DVTECNSNNG	.....	DEQAISNKCC	AHPNGCTLDYIQRWC	CRNENKYDVV
***As*-INS-novel2**	MLQLKGDQHI	KACGAHLEAIMNSVC	SYGNHKLPCY	.....	SSESAVIVSK	CCNVWCTKKEIERVC	CFTEQCLKKC

Fig. 9.2. Motifs and conserved pattern of insulin-like peptides inferred for *Caenorhabditis elegans*, *Toxocara canis* and *Ascaris suum*. Motifs RLCGRKLIKAVQSLC and CCSKGCTDEDJKKYC in the inferred protein sequences are aligned and indicated in sequence logos. The conserved pattern C-C in these sequences is indicated in blue.

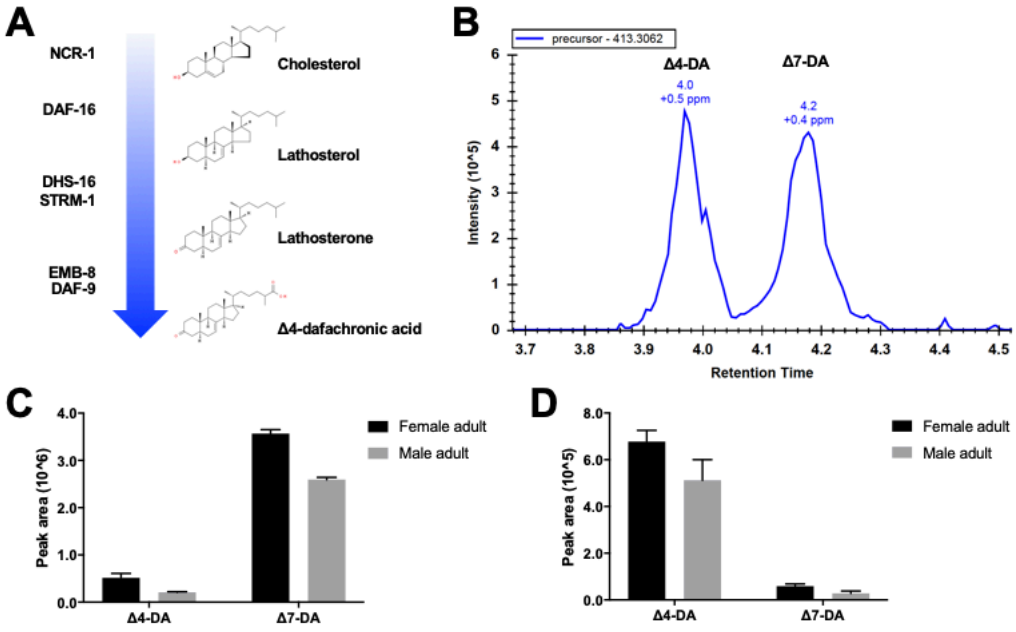


Fig. 9.3. Dafachronic acids (DAs) in *Toxocara canis* and *Ascaris suum*. (A) Proposed biosynthetic pathway of Δ7-DA in *Toxocara canis* and *Ascaris suum*, which involves NCR-1, DAF-36, DHS-16, STRM-1, EMB-8 and DAF-9. (B) Using Δ4- and Δ7-DAs (exact mass = 413.3061) as standards, endogenous DAs, with mass errors estimated at 0.5 and 0.4 parts per million (ppm), are present in the adults (female and male) of *Toxocara canis* and *Ascaris suum*, respectively. (C) and (D) Relative abundances of Δ4- and Δ7-DAs in the adult (female and male) stages of *Toxocara canis* and *Ascaris suum*, respectively.

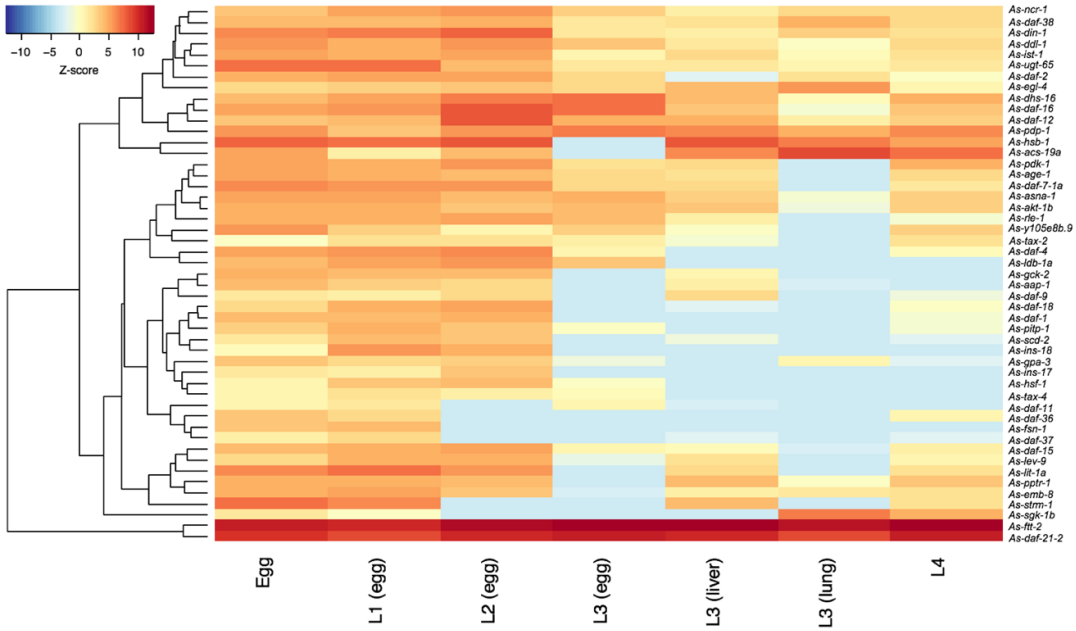


Fig. 9.4. Transcriptional profiles of dauer signalling genes in different developmental stages of *Ascaris suum*. Transcriptional levels (transcripts per million, TPM) of dauer signalling orthologues in egg, the first- [L1 (egg)] and second-stage larvae [L2 (egg)] recovered from eggs; third-stage larvae from eggs [L3 (egg)], host liver [L3 (liver)] or lung [L3 (lung)], and the fourth-stage larvae (L4) of *Ascaris suum* are indicated in the heat map. Colour scales and Z-scores indicate scaled TPMs.

Supplementary Table S9.1. Dauer signalling gene homologues in *Toxocara canis* and *Ascaris suum*.

Supplementary Table S9.2. Orthologous groups of dauer signalling genes in *Toxocara canis* and *Ascaris suum* inferred from *Caenorhabditis elegans*.

Supplementary Table S9.3. Salient information on dauer signalling genes of *Toxocara canis* predicted from *Caenorhabditis elegans* orthologues.

Supplementary Table S9.4. Salient information on dauer signalling genes of *Ascaris suum* predicted from *Caenorhabditis elegans* orthologues.

Supplementary Table S9.5. Classification of insulin-like peptide homologues in *Toxocara canis* and *Ascaris suum*.

Supplementary Table S9.6. Transcription of dauer-like signalling gene orthologues in key developmental stages of *Toxocara canis* and *Ascaris suum*.

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Chapter 10. General discussion

A critical review of the literature (Chapter 1) showed that there are major gaps in our knowledge and understanding of developmental processes in parasitic nematodes, and indicated that filling some of these gaps using advanced technologies could hold a key to discovering innovative approaches to control socioeconomically important nematodes of animals. With this focus in mind, the present thesis set out: (i) to establish comprehensive transcriptomic, proteomic and lipidomic resources for *H. contortus* (Chapters 2-5); (ii) to explore molecular alterations occurring during the developmental transition of this nematode (Chapter 5); (iii) to gain detailed insights into the signalling pathways involved in the development of *H. contortus* (Chapter 6); and (iv) to elucidate the functionality of the dauer-like signalling pathway and the potential role(s) of bile acid-like dafachronic acids in governing parasite development (Chapters 7-9). These goals were all achieved, and the research outcomes indicated some unique avenues for future molecular investigations of parasitic nematodes.

The purpose of the present chapter is: (a) to contextualise key research achievements; (b) to discuss, in a general context, the research results and implications in relation to the developmental biology of *H. contortus* and other parasitic worms; and (c) to provide a perspective on the challenges and opportunities arising from this work in the field of molecular parasitology in a relatively broad framework.

10.1. Integrated genomic, transcriptomic, proteomic and lipidomic studies provide a paradigm of multi-omics

Transcriptomic (mRNAs and miRNAs), proteomic (somatic, excretory/secretory and phosphorylated proteins) and lipidomic (polar and neutral lipids) data resources were established for *H. contortus* (see Chapters 2-5). Together with the draft genomes (Laing et al., 2013; Schwarz et al., 2013; Doyle et al., 2018), these data sets now represent the most comprehensive molecular resources for *H. contortus*. Analyses of the transcriptomic, proteomic and lipidomic data sets for *H. contortus* revealed significant differences in the profiles of mRNAs, proteins and lipids among developmental stages,

which were associated with a range of biological processes, such as metabolism (carbohydrate, lipid and amino acid metabolisms), environmental adaptation and parasitism. However, separate transcriptomic, proteomic and lipidomic studies hindered somewhat the interpretations of research findings. Therefore, in Chapter 5, integrated transcriptomic (mRNAs and miRNAs) and proteomic (somatic proteins) analyses ('multi-omics') of *H. contortus* from precisely the same samples revealed molecular alterations during developmental processes in this worm, which enabled the discovery of post-transcriptional regulation by miRNAs (e.g., *hco-miR-34* and *hco-miR-252*) and inferred chemosensory signal transduction in larval developmental transition of this parasitic nematode. Compared with previous analyses of either mRNAs, miRNAs *or* proteins of *H. contortus* (e.g., Cantacessi et al., 2010; Winter et al., 2012; Wang et al., 2016), the multi-omics approach (Chapter 5) showed major advantages in identifying molecular alterations and signalling pathways linked to the developmental processes in this nematode (Fig. 10.1). This work has provided a unique basis for profound molecular investigations of this and related parasitic nematodes.

10.2. Exploring dauer-like signalling in *H. contortus* using multi-omics

Having available the genomic, transcriptomic, proteomic and lipidomic resources and tools in place allowed in-depth investigations of *H. contortus*. For instance, an integrated multi-omic study (Chapter 5) discovered that steroid hormone biosynthesis and hormone signal transduction likely play crucial roles in the larval activation and development in *H. contortus*. Although little was known and understood about steroid hormones and signal transduction in this worm (Nikolaou and Gasser, 2006), the 'dauer hypothesis', positing that the infective larva of a parasitic nematode represents a dauer-constitutive analogue of a free-living nematode (cf. Hotez et al., 1993; Viney, 2009; Crook, 2014) provided a conceptual framework to start to understand this area. Specifically, in this thesis (Chapter 6), using comparative genomic, transcriptomic and proteomic approaches, the cyclic guanosine monophosphate, transforming growth factor β, insulin/insulin-like growth factor 1 and steroid hormone signalling pathways - collectively the "dauer signalling pathway", known to govern the dauer formation in the free-living nematodes *Caenorhabditis elegans* (see Hu, 2007; Fielenbach and Antebi, 2008) - were

reconstructed for *H. contortus*. This reconstructed "dauer-like" signalling pathway model for *H. contortus* allowed explorations of other parasitic and free-living nematodes. For example, a comparative transcriptomic and proteomic investigation of the L3 stage of *H. contortus* and the dauer stage of *Caenorhabditis elegans* revealed relatively concordant mRNA transcription and protein phosphorylation profiles for homologous molecules (Chapter 6). Despite this similarity, some distinctions were found in the number of gene homologues coding for G protein-coupled receptors (GPCRs), insulin-like peptides and cholesterol transporters between the two species.

First, the apparent absence of gene homologues encoding GPCRs (SRBC-64 and -66; and SRG-36 and -37) from *H. contortus* might represent an adaptation to a relatively stable food source and temperature within the host animal. This proposal might be supported by a reduced number of GPCRs in parasitic nematodes of different groups, such as *Trichinella* and *Trichuris* (clade I), *Ascaris*, *Brugia* and *Onchocerca* species (clade III) (cf. International Helminth Genomes Consortium, 2019), which may not require extensive chemosensory behaviour in response to environmental changes outside of the host animal. Even smaller numbers of chemosensory GPCRs were identified in platyhelminths including *Clonorchis sinensis*, *Fasciola hepatica* and *Schistosoma* species (see Zamanian et al., 2011; McVeigh et al., 2018; International Helminth Genomes Consortium, 2019), but it is presently unclear whether this finding relates to evolution/host adaptation or to the fragmentation of current draft genomes, thus requiring further evaluation. In addition, the absence of genes coding for the ascaroside receptor proteins SRBC-64, -66 and SRG-36, -37 in *H. contortus* might also suggest a loss of ascarosides ascr#2, #3 and #5 from this parasitic nematode. This proposal is supported somewhat by the reduced number of ascarosides detected in the infective and adult stages of *Nippostrongylus brasiliensis* (see Choe et al., 2012). A large-scale investigation of ascarosides in different developmental stages of parasitic nematodes of animals would stimulate the discovery of corresponding GPCRs and could lead to a better understanding of ascaroside signalling in parasitic worms.

Second, the exclusive gene homologues coding for antagonistic insulin-like peptides in *H. contortus* might associate with the programmed larval diapause in infective larvae in the environment outside of the host animal. This statement is strongly supported by high transcriptional levels of *ins-1*, *ins-17* and *ins-18* seen in the L3 stage of *H. contortus*

(Chapters 6 and 7), which is also observed during dauer-formation in *Caenorhabditis elegans* (see Pierce et al., 2001; Matsunaga et al., 2012a,2012b). In addition, an apparent loss of gene homologues (*daf-28*, *ins-4* and *ins-6*) coding for agonistic insulin-like peptides in *H. contortus* might also contribute to programmed larval diapause, as these homologues are known to promote larval development in *Caenorhabditis elegans* (see Zheng et al., 2018; Kaplan et al., 2019). The lack of gene homologues encoding agonistic insulin-like peptides suggests potential roles of host insulin in the developmental processes in parasitic nematodes within their host animal(s). This proposal might be evaluated in *H. contortus* using functional genomic and/or developmental assays and using an in vitro ligand-binding assay (in which host insulin binds to the parasite insulin receptor).

Third, the reduced number of gene homologues coding for cholesterol transporters in *H. contortus* might relate to a stable source of nutrients in/from the host. Specifically, the exclusive gene homologue *Hc-ncr-1* is proposed to play a role in the uptake of cholesterol from dietary bacteria in the free-living larval (L1 and L2) stages of *H. contortus*, similar to the situation in *Caenorhabditis elegans* (see Sym et al., 2000; Li et al., 2004; Watts and Ristow, 2017). This gene homologue might also play a role in the utilisation of host cholesterol, as it is a homologue of the gene (*npc-1*) involved in intracellular cholesterol trafficking in mammals (Watari et al., 1999). The reduced gene number in *H. contortus* might relate to an adaptation to a rich source of cholesterol in the host animal. Gene knockdown or knockout evaluations of this gene homologue in *H. contortus* might be able to provide direct evidence for this proposal.

Given the implied divergence in the numbers of gene homologues coding for GPCRs, insulin-like peptides and cholesterol transporters between free-living and parasitic nematodes (cf. Stoltzfus et al., 2012; Albarqi et al., 2016; Gilabert et al., 2016), it would be interesting to explore similarities and differences in dauer-like signalling between genetically divergent strains of *H. contortus* with mating barriers between allopatric strains (Sargison et al., 2019). The dauer-like signalling pathway model constructed in this thesis (Chapter 6) provides a basis to explore evolutionary and functional aspects of chemosensation, steroid hormone biosynthesis and hormone signal transduction during developmental processes in *H. contortus* and other parasitic nematodes. An improved understanding of these areas would fill some of the existing gaps in our knowledge of the

developmental biology of parasitic nematodes within their host animal(s), including, for example, the effects and mechanisms of host factors (e.g., bile acid) on larval activation, development, motility and/or establishment (cf. Jacqueline et al., 1981; Mapes, 1972; Han et al., 2000).

10.3. Integrated multi-omics elucidated roles of a bile acid-like hormone in development processes in *H. contortus*

A bile acid-like (dafachronic acid) signalling pathway was elucidated in *H. contortus* using the multi-omics approach established (Chapter 7). Specifically, an upregulation of the endogenous biosynthesis of Δ7-dafachronic acid was detected following the transcriptional activation of genes involved in the dauer-like signalling pathway. The Δ7-dafachronic acid signal was linked to larval exsheathment and development in vitro, and a downregulation of mRNA transcription and protein expression via the nuclear hormone receptor DAF-12 during the developmental transition from the free-living to the parasitic stages of *H. contortus*. These molecular changes linked to the modulation of metabolism of odd-chain lipid species (glycerolipids and glycerophospholipids). Although further study is required to understand the roles of odd-chain lipid metabolism in signal transduction, the experimental results indicated important roles for steroid hormone signal in the developmental transition in *H. contortus*. Interestingly, the dafachronic acid signal appears to have distinct effects on the developmental processes of obligate and facultative parasitic nematodes (cf. https://biologydictionary.net/parasitism/; Crook, 2014). Specifically, in 'obligate' *H. contortus*, the hormone was shown to promote the transition from the free-living, infective stage to the parasitic stage (Chapter 7), whereas, in 'facultative' *Strongyloides stercoralis*, this hormone signal was found to prevent the formation of infective larvae and stimulate the switch to the free-living phase (Albarqi et al., 2016). Therefore, although dafachronic acid can suppress the hyperinfection of *Strongyloides stercoralis* in highly immune-compromised mice (Patton et al., 2018), such an intervention strategy is unlikely to work for *H. contortus* and related parasitic nematodes (e.g., *Ancylostoma* species). However, an interruption of the endogenous biosynthesis of dafachronic acid inhibited larval activation and development of *H.*

contortus in vitro (Chapter 7), suggesting that an alternate, novel intervention strategy could be established for this and possibly for related parasitic nematodes.

The inferred bile acid-like signalling pathway in *H. contortus* suggests key roles for host bile acids in larval activation and development. This statement is supported to some extent by the structural similarities of dafachronic acid and particular metabolite (e.g., cholestenoic acid) of host bile acid (Held et al., 2006; Motola et al., 2006; Zhi et al., 2012), although cholestenoic acid does not induce a resumption of feeding in infective larvae of a related nematode (*Ancylostoma ceylanicum*) (see Zhi et al., 2012). Nevertheless, the dafachronic acid-DAF-12 signalling module in *H. contortus* would appear to provide a paradigm to understand the roles of host factors in site selection by nematodes (e.g., *Heligmosomoides polygyrus*; see Sukhdeo et al., 1988) and host specificity in other helminths (e.g., the tapeworm *Echinococcus granulosus*; see Smyth and Haslewood, 1963). A deep understanding of chemosensation of and signal transduction in parasitic worms would likely provide insights into the physiochemical communication between parasite and host animal (cf. Lok, 2016), and could enable the discovery of novel anthelmintic targets.

10.4. Signalling pathway components as potential drug targets

The lack of genes encoding agonistic insulin-like peptides, the orphan gene (*Hc-ncr-1*) encoding a cholesterol transporter and the crucial roles of bile acid-like hormone signalling in *H. contortus* (Strongylida), *Ascaris suum* and *Toxocara canis* (Ascaridida) (Chapters 6,7,9) suggest novel targets for anthelmintics.

Receptors that bind host insulin might represent such a target in parasitic nematodes. Given that no gene homologue encoding an agonistic insulin-like peptide was identified in either *H. contortus*, *Ascaris suum* or *Toxocara canis* (Chapters 6 and 9), host insulins might serve as a complementary signal for the activation of infective larvae within the host animal. This statement is somewhat supported by previous findings that muscarinic agonists increase *Caenorhabditis elegans* insulin-like signalling and induce the activation of arrested larvae of *Ancylostoma caninum* (see Tissenbaum et al., 2000). The function or essentiality of gene homologues encoding the insulin receptor of members of the nematode orders Strongylida and Ascaridida could be assessed using gene perturbation

assays. Moreover, targeting the agonistic insulin-like peptide receptor with specific inhibitors might represent a novel intervention strategy.

Apart from the insulin receptor, the cholesterol transporter in *H. contortus*, *Ascaris suum* and *Toxocara canis* might represent another target. Considering the deficiency of parasitic nematodes to synthesise cholesterol and glycolipids (cf. Behm, 2002; Vinci et al., 2008), the inferred orphan cholesterol transporter in these parasitic nematodes likely plays a significant role in cholesterol uptake from host sources. Interrupting the uptake of extracellular cholesterol from host tissues or fluids might block biological processes in parasitic nematodes, compromising the development and/or survival of the worms within the host animal. Nematode-specific binding proteins (e.g., fatty acid- and retinoid-binding proteins and nematode polyprotein allergens) should also be evaluated as target candidates, as they appear to have roles in lipid uptake from the host animal (cf. McDermott et al., 1999; Fairfax et al., 2009; Kennedy, 2011).

In addition, the disruption or interruption of the bile acid-like dafachronic acids signalling pathway might represent a promising intervention approach, as bile acid-like dafachronic acids play a critical role in developmental transition in *H. contortus* (see Chapter 7) and other parasitic nematodes (Gerisch and Antebi, 2004; Wang et al., 2009; Bento et al., 2010; Albarqi et al., 2016). Compounds that specifically inhibit the endogenous biosynthesis of dafachronic acids and steroid hormone signalling in parasitic nematodes could represent anthelmintic candidates (cf. Rottiers et al., 2006; Wang et al., 2017). For instance, dafadine A (inhibitor of DAF-9/cytochrome P450; Luciani et al., 2011) inhibits the biosynthesis of dafachronic acids and suppresses larval activation in *H. contortus* (Chapter 7), and small interfering RNA (siRNA)-mediated suppression of the nuclear hormone receptor DAF-12 perturbs larval development in *Strongyloides ratti* (see Dulovic and Streit, 2019).

Clearly, the druggability of the insulin receptor, cholesterol transporter, DAF-9 and DAF-12 requires evaluation in the future. In particular, the efficiency and/or toxicity of compounds targeting these molecules, such as chemical inhibitors or synthetic siRNAs, need to be comprehensively assessed in vivo in the host animal. Functional genomics (by gene knockdown and/or knockout) of *H. contortus* and related parasitic nematodes, comparative metabolomic studies between parasitic nematodes and host animal tissues, and in vivo studies of nematode development could contribute to the identification of

other receptors in parasitic nematodes and corresponding ligands from the host (e.g., sex steroids) (Ganley-Leal, 2005; Jin et al., 2008; Hernández-Bello et al., 2011; Mahanti et al., 2014), and improve our understanding of host factor-effects on the larval activation and development of nematodes within host animals. A better knowledge of the signalling pathways involved in the developmental processes of such worms should stimulate the discovery of novel targets for anthelmintic drugs.

10.5. Future work

Apart from the insights into the developmental biology of *H. contortus* (sections 11.1.-11.4.), a multi-omics approach could underpin future research in a number of areas, such as the evolution and parasitism of helminths, host-parasite interactions, and the pathogenesis of disease.

The dauer-like signalling and bile acid-like signalling pathways model proposed in this work may be relatively conserved among parasitic nematodes, trematodes and cestodes. Specifically, the dauer-like signalling pathways model in *H contortus*, *Ascaris suum* and *Toxocara canis* were proposed based on the dauer hypothesis, which posits that the dauer formation in free-living nematodes is a pre-adaptation to parasitism (Viney, 2009; Crook, 2014; Sommer and Ogawa, 2011). This seems clear for the suspended development (diapause) of free-living, infective larvae of parasitic nematodes representing the orders Ascaridida, Rhabditida and Strongylida, as they share some physiological similarities with the dauer larvae of the free-living nematode *Caenorhabditis elegans*. However, there are other forms of arrested development in parasitic nematodes occurring in host animals: e.g., (i) hypobiosis of the fourth-stage larvae of species of *Ostertagia, Teladorsagia*, *Haemonchus* and *Cooperia* of ruminants (reviewed by Michel, 1974; Gibbs, 1986); (ii) arrested development of the tissue-migrating larvae of *Ancylostoma*, *Baylisascaris* and *Toxocara* species in paratenic hosts (Michel, 1974; Gibbs, 1986; Strube et al., 2013); and (iii) suspended development of the infective larvae of *Brugia malayi*, *Dirofilaria immitis*, *Loa loa* and *Onchocerca volvulus* in intermediate hosts. The hormonal signalling mechanism might play roles in these different forms of developmental arrest and/or in the activation of parasitic larvae in response to host conditions or factors, such as immune responses, hormone levels, and/or

parasite factors (Beckage, 1991; Beall and Pearce, 2002; Viney et al., 2005; Strube et al., 2007; Ogawa et al., 2009; Lok, 2016). Beyond nematodes, the chemosensory signalling pathways controlling development likely exist in trematodes and cestodes as well. For instance, the roles of GPCRs and insulin-like peptides in the development of *Schistosoma mansoni* and *Echinococcus multilocularis* have been indicated (Hoffmann et al., 2001; Hemer et al., 2014; Du et al., 2019). Although there is a lack of information about these aspects, evidence for crustaceans, insects and fishes (e.g., Hand et al., 2016; Mathew et al., 2017; Romney et al., 2018) suggest similar molecular mechanisms in a wide range of animals. Multi-omics studies of key worm species whose genomes are known, such as *Schistosoma mansoni*, *Echinococcus granulosus*, *Echinococcus multilocularis* and *Taenia multiceps* (see Berriman et al., 2009; Protasio et al., 2012; Tsai et al., 2013; Zheng et al., 2013; Li et al., 2018; ParaSite: https://parasite.wormbase.org/species.html), could be conducted to elucidate signal transduction (pheromone and hormone signalling) pathways in these helminths and their molecular biology.

A better knowledge of pheromone and hormone signalling could shed a light on chemosensation by helminths during tissue migration and on organ tropism. For instance, the infective larvae of some nematodes, such as *Angiostrongylus*, *Baylisascaris* and *Toxocara* species, undertake tissue migration within paratenic host animals, and the migrating larvae tend to invade the central nervous system and somatic tissues of paratenic hosts (Kazacos et al., 2013; Strube et al., 2013; Janecek et al., 2014). In addition to nematodes, trematodes such as *Fasciola hepatica* and *Clonorchis sinensis* dwell as adults in the bile ducts of the liver of the definitive host, whereas larval stages of cestodes, such as *Echinococcus granulosus* and *Taenia solium*, tend to invade liver and lung parenchyma or brain of the intermediate or accidental host animal. Thus, pheromone and hormone signalling might play roles in organ/tissue tropism and establishment in, or migration through, particular tissues (Safer et al., 2007; Hallem et al., 2011; Chaisson and Hallem, 2012). Although pheromones and hormones have been identified in selected parasitic nematodes of animals, such as *Ascaris lumbricoides* and *Nippostrongylus brasiliensis* (see Bartley et al., 1996; Choe et al., 2012; Butcher, 2017), very little is known about these molecules in socioeconomically important helminths, such as species of *Angiostrongylus* and *Toxocara* (nematodes), and *Clonorchis* and *Echinococcus* (platyhelminths). Integrative ‘omic studies of these pathogens as well as their host

animals (particularly at the host-parasite interface) could elucidate aspects of organ and tissue tropism of parasitic nematodes, trematodes and cestodes at the molecular level.

Using multi-omics for studies of the host-parasite interface could also provide insights into the pathogenesis of disease. Recent evidence has indicated that exosomes, a group of membranous vesicles released by helminths during infection, might play roles in host-parasite interactions (Maizels et al., 2018). These small vesicles contain nucleotides (mRNA and miRNAs), proteins (excretory/secretory molecules) and lipids, some of which are well known for their roles in immune recognition and evasion (Buck et al., 2014; Maizels et al., 2018). However, although exosomes/exosome-like vesicles have been isolated and characterised in nematodes such as *Heligmosomoides polygyrus* (see Buck et al., 2014), *Brugia malayi* (see Zamanian et al., 2015), and trematodes including *Echinostoma caproni*, *Fasciola hepatica* (see Marcilla et al., 2012) and *Schistosoma japonicum* (see Zhu et al., 2016), the contents of these exosomes/exosome-like vesicles have not yet been comprehensively defined. Defining the components of exosomes/exosome-like vesicles released from species and/or stages of helminths using advanced RNA-seq, mass spectrometry-based proteomics and lipidomics could elucidate the intricate mechanisms or processes involved in host-parasite 'cross-talk' (Swann et al., 2015). A better knowledge of exosomal components would also contribute to applied areas, as they might represent biomarkers for diagnosis and/or for drug resistance in helminths (Britton et al., 2014; Tritten et al., 2014; Gillan et al., 2017). Advances in the in vitro co-culture of parasitic stages of helminths and host cells (e.g., intestinal epithelial cells or intestinal organoids) (Feather et al., 2017; Dutta and Clevers, 2017) could also enable multi-omic investigations of processes that occur at the host-parasite interface.

10.6. Challenges and opportunities

Multi-omics provides an avenue for in-depth systems biology investigations (cf. Kitano, 2002; Ge et al., 2003). Data integration needs to tackle the principal challenges of producing high-resolution and reproducible data sets and, importantly, to make biological meaning from the data sets employing reliable in silico and in vitro tools.

Although advances have been achieved in the genomics, transcriptomics and proteomics of helminths (e.g., Stoltzfus et al., 2016; International Helminth Genomes

Consortium, 2019; Jex et al., 2019), many molecular components (e.g., genes, transcripts, proteins, lipids and carbohydrates) have not yet been comprehensively characterised or defined in worms. The availability of draft genomes and extensive transcriptomes does lay a foundation for mass spectrometry-based proteomic and lipidomic studies, but there are still difficulties in identifying and defining all proteins and lipids using current bioinformatic and mass spectrometry-based technologies (Korhonen et al., 2016). For instance, transcription, expression or abundance can be low for some molecules (McNulty et al., 2012; Luck et al., 2015), beyond the limits of detection by conventional methods (Olsen and Mann, 2013; Ke et al., 2016). High-throughput sequencing of full-length transcripts (e.g., PacBio and Oxford Nanopore technologies), enrichment techniques for low-abundance molecules (e.g., TiO_2-enrichment and extraction methods for nonpolar lipids), specialised mass spectrometry-based proteomic and lipidomic analyses as well as advanced data integration and visualisation algorithms (Hwang et al., 2005; Draghici et al., 2007; Gehlenborg et al., 2010; Renuse et al., 2011; Nesvizhskii, 2014) are required for comprehensive identification and detection. Very little progress has been made in identifying carbohydrates, lipids and metabolites of parasitic nematodes. Progress in these fields requires well-established and -curated databases, high-throughput platforms and reliable data processing algorithms. Establishing comprehensive multi-omics resources (pertaining to genome, transcriptome, proteome, glycome, lipidome and metabolome) for parasitic nematodes would require considerable investment and efforts. Multi-omics and 'systems biology' investigations of model organisms such as *Caenorhabditis elegans* (see Gaertner and Phillips, 2010; van Assche et al., 2015) and *Pristionchus pacificus* (see Borchert et al., 2012; Sommer and McGaughran, 2013) provide platforms to support future explorations of socioeconomically important parasitic worms.

Most genomic and transcriptomic studies of parasitic worms have usually been conducted using pools of individuals of one or multiple developmental stages. Such pools do not actually represent the tissues and cells within these stages (cf. Wang et al., 2017; Hahnel et al., 2018). Thus, the transcriptomic data from one or pooled individual(s) cannot provide accurate information about transcriptional and biological changes in specific tissues or cells. For instance, it was found that the polarity of insulin-like peptides secretion determines their functional roles in larval diapause in *Caenorhabditis elegans*

(see Matsunaga et al., 2016), which cannot be revealed using conventional transcriptomic studies of whole organisms or pools thereof. Therefore, tissue-specific transcriptomic and proteomic studies are required to provide high-resolution molecular data. Single cell sequencing, for example, has been successfully applied to *Caenorhabditis elegans*, which successfully defined expression profiles for different cell types ($n > 20$) and discovered rare cell types (Cao et al., 2017), which is simply not achievable using conventional methods. Clearly, single cell multi-omics (Grün and van Oudenaarden, 2015; Macaulay et al., 2017) provides opportunities for genuine 'systems biological' investigations of the tissues and cells of parasitic nematodes, trematodes and cestodes.

Without functional information about genes, RNAs and proteins, the value of results from 'omics studies is compromised. For example, comparative genomics or transcriptomics has revealed horizontal gene transfer and gene loss, expanded gene families coding for astacin-like and SCP/TAPS proteins, conservation and diversity of genes coding for GPCRs, kinases and small RNAs in parasitic nematodes (Dieterich and Sommer, 2009; Winter et al., 2012; Ahmed et al., 2013; Hunt et al., 2016, 2017; Stroehlein et al., 2017). However, there is almost no functional information about these unique and/or orphan (unknown) genes in parasitic nematodes. This paucity relates to a lack of effective functional genomic platforms for parasitic worms. Various studies have shown that long double-strand RNA (dsRNA), small interfering RNA (siRNA) or short-hairpin RNA (shRNA)-mediated RNA interference (RNAi) approaches might not work effectively and reproducibly in parasitic nematodes (Geldhof et al., 2007; Britton et al., 2016). Transgenesis and genome editing (e.g., clustered regularly interspaced short palindromic repeats and associated nuclease Cas9 system, CRISPR/Cas9; Jinek et al., 2012) as well as retrovirus-based silencing (lentivirus-based RNAi) seem to be promising for helminths (e.g., Lok, 2012; Friedland et al., 2013; Hagen et al., 2014; Britton et al., 2016; Sugi, 2016; Gang et al., 2017; Ward, 2018), but are not widely employed, because of the challenges associated with their establishment, optimisation and application. Establishing and consolidating some of these advanced tools in the field of parasitology would be a major step forward for functional genomics of parasitic worms.

10.7. Conclusions

This thesis has yielded comprehensive transcriptome (mRNA and miRNA), proteome (somatic, secretory and phosphorylated proteins) and lipidome data sets, which will be of major benefit as resources to the scientific community working on parasitic nematodes including *H. contortus*. On the other hand, the integrative, multi-omics approach established has given comprehensive insights into selected aspects of the developmental biology of *H. contortus*, and, in my opinion, provides an advancement for exploring the systems biology of many other parasitic nematodes (Fig. 10.1). This multi-omics 'systems biology' approach will hopefully stimulate similar molecular biological investigations of socioeconomically important trematodes and cestodes, and might contribute to discovering novel interventions in the medium- to long-term. Although applied here to a parasitic nematode system, this multi-omics concept can also readily be used to explore the molecular biology of other pathogens, such as protozoa, bacteria, viruses and fungi, as well as their interactions with their hosts. Such an approach will likely significantly improve the understanding of pathogens and infectious diseases, and could pave the way to new diagnostic and intervention strategies.

10.8. References

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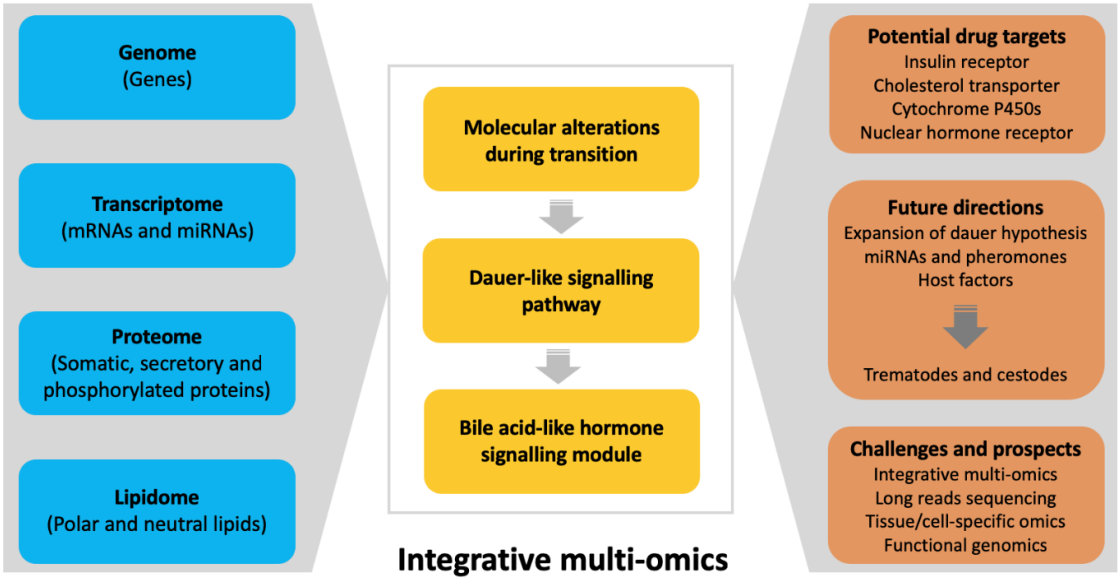


Fig. 10.1. Integrative multi-omics approach (blue) established in this thesis for investigating aspects of developmental biology of *Haemonchus contortus* (yellow), and its implications for future fundamental and applied research directions, extending beyond this thesis (orange).