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Transcriptomic and proteomic analysis of *Ascaris suum* larvae during their hepato-tracheal migration

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Table of contents

CHAPTER ONE

Introduction 9

1.1 Life cycle 11

1.2 Epidemiology 14

1.2.1 Prevalence of A. lumbricoides infection 14

1.2.2 Prevalence of A. suum infection 15

1.3 Pathology & Economic loss 19

1.3.1 A. lumbricoides 19

1.3.2 A. suum 19

1.4 Immunity development against infections with Ascaris 24

1.5 Control strategy 25

1.5.1 Control strategies of human ascariasis in practice 25

1.5.2 Control strategies of pig ascariasis in practice 27

1.5.3 Problems and limitations in the current control strategies against ascariasis 31

1.5.4 Alternative control strategy of ascariasis – vaccination 32

1.6 Concluding thoughts 38

OBJECTIVES 41

CHAPTER TWO

Transcriptomic analysis of Ascaris suum larvae during their hepato-tracheal migration 43

2.1 Introduction 44

2.2 Materials and Methods 45

2.2.1 Ethics Statement 45

2.2.2 Parasite material 45

2.2.3 RNA isolation, sequencing and assembly 45

2.2.4 Differential transcription analysis 46

2.2.5 Quantitative real-time PCR 46

2.2.6 Functional annotation of coding genes 47

2.2.7 Molecular mimicry survey 47

2.3 Results and Discussion 47

2.3.1 A. suum larval transcriptome datasets 47

2.3.2 Top 250 most abundant transcripts in the different larval stages 48

2.3.3 Stage specific gene transcription ____ **54**

2.3.4 Metabolic pathways ____ **60**

2.3.5 Chemosensory pathways ____ **61**

2.3.6 Molecular mimicry ____ **63**

CHAPTER THREE

Proteomic analysis of the excretory-secretory products from larval stages of Ascaris suum ____ **66**

3.1 Introduction ____ **67**

3.2 Materials and Methods ____ **67**

3.2.1 Ethics Statement ____ **67**

3.2.2 Parasite material ____ **68**

3.2.3 Preparation and analysis of ES products ____ **68**

3.2.4 In-gel and in-solution tryptic digestion and LC-MS/MS analysis ____ **69**

3.2.5 Database searching and sequence analysis ____ **70**

3.2.6 RNA extraction and quantitative real-time PCR (qPCR) ____ **71**

3.2.7 Analysis of differential transcription ____ **72**

3.2.8 Protein extraction and enzymatic assays ____ **72**

3.3 Results ____ **73**

3.3.1 Proteins profiles of the excretory/secretory material ____ **73**

3.3.2 Protein identifications ____ **74**

3.3.3 Glycosyl hydrolases in Ascaris suum ____ **81**

3.4 Discussion ____ **86**

CHAPTER FOUR

Proteomic analysis of the cuticle surface associated proteins from the infective stage larvae of Ascaris suum ____ **92**

4.1 Introduction ____ **93**

4.2 Materials and Methods ____ **94**

4.2.1 Parasite material ____ **94**

4.2.2 Identification of surface associated proteins through biotinylation ____ **94**

4.2.3 Identification of surface associated proteins by enzymatic shaving ____ **96**

4.2.4 Database searching and sequence analysis ____ **97**

4.3 Results ____ **98**

4.3.1 Identification of surface associated proteins through biotinylation ____ **98**

4.3.2 Identification of surface associated proteins by enzymatic shaving ____ **102**

4.4 Discussion _____ **104**

CHAPTER FIVE

General discussion and conclusions _____ **108**

5.1 Identification of Ascaris suum excretory-secretory proteins _____ **109**

5.2 Identification of surface associated proteins of Ascaris larvae _____ **110**

5.3 Potential Ascaris drug- and vaccine targets identified by the proteomic and transcriptomic analyses _____ **110**

5.3.1 Glycosyl hydrolases _____ **110**

5.3.2 C-type lectin _____ **112**

5.3.3 14-3-3 protein _____ **114**

5.3.4 Transthyretin-like protein _____ **115**

5.4 Conclusions _____ **116**

SUMMARY _____ **117**

List of figures

Figure 1.1 Life cycle of *A. suum* in pigs
Figure 1.2: Mophology of different stages of *A. suum*
Figure 1.3 The global distribution of *A. lumbricoides* infections
Figure 1.4 The countries with available prevalence information of *A. suum*
Figure 1.5 The globe distribution of DALYs for ascariasis
Figure 2.1 Molecular function distributions of the top 250 abundant transcripts
Figure 2.2 Molecular function distributions of the top 50 specific transcripts
Figure 2.3 The RPKM values ratio of the genes involved in different metabolism
Figure 2.4 ClustalW aligment of *A. suum* GS_13355 to SOCS5 from *Sus domesticus*
Figure 3.1 Protein profiles of the *A. suum* L3-egg, L3-lung and L4 ES products
Figure 3.2 The distribution of the number of identified larval ES proteins
Figure 3.3 Molecular function assigned to the identified larval ES proteins
Figure 3.4 Phylogenetic tree and signature motifs of GH31 proteins
Figure 3.5 qPCR and glucosidase hydrolytic activity analysis of GH31 proteins
Figure 4.1 Approach for the identification of surface proteins in *A. suum* larvae.
Figure 4.2 Microscopical analysis of the biotinylated- and control *A. suum* larvae
Figure 4.3 SDS-PAGE gel separation of bound material and corresponding blots.
Figure 5.1 The RPKM values ratio of the genes involved in starch metabolism

List of tables

Table 1.1 Clinical features and general outcomes associated with *ascariasis*
Table 1.2 Efficacy of anthelminthic drugs tested against *Ascaris*
Table 1.3 Vaccination studies performed against *ascariasis*
Table 2.1 Nucleotide sequences of the primers used in the qRT-PCR assays
Table 2.2 Characteristics of the expressed sequence tags (ESTs)
Table 2.3 List of cuticle collagens identified in the top 250 abundant transcripts
Table 2.4 Top 20 KAAS pathways of abundant proteins from *A. suum* larvae
Table 2.5 List of *C. elegans* chemosensory homologues in *A. suum* dataset
Table 3.1 Nucleotide sequences of the primers used in the qRT-PCR assays
Table 3.2 Protein identifications in *A. suum* L3-egg ES products
Table 3.3 Protein identifications in *A. suum* L3-lung ES products
Table 3.4 Protein identifications in *A. suum* L4 ES products
Table 3.5 List of glycosyl hydrolases identified in the *A. suum* genome
Table 4.1 The identified surface associated proteins from the *A. suum* larvae
Table 4.2 The identified surface and released proteins from the *A. suum* larvae

List of abbreviations

AA	amino acids
ASABF	*Ascaris suum* antibacterial factor
BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
cGMP	3'-5'-cyclic guanosine monophosphate
CT-B	Cholera Toxin subunit B
DALYs	disability-adjusted life years
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
emPAI	Exponentially Modified Protein Abundance Index
ES	excretory-secretory
EST	expressed sequence tag
FA	formic acid
FCA	Freund's complete adjuvant
FIA	Freund's Incomplete Adjuvant
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH31	glycosyl hydrolases family 31
GO	Gene Ontology
i.e.	id est
Ig	immunoglobulin
IL	Interleukin
kDa	kilo Dalton
L3	third stage larva
L4	fourth stage larva
LC	liquid chromatography
M	Molar
ML	macrocyclic lactone

MS	mass spectrometry
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
p.i.	post infection
RNA	ger ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPKM	reads per kilobase per Million mapped reads
SB3-10	3-(Decyldimethylammonio)propanesulfonate inner salt
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp.	species
SOCS	suppressor of cytokine signaling family
STHs	soil-transmitted helminthes
Th2	Type 2 helper T cells
UV	Ultraviolet

CHAPTER ONE

Introduction

Ascaris lumbricoides, Linnaeus, 1758 of humans and *A. suum*, Goeze, 1782 of swine are the largest and the best-known roundworms inhabiting the intestine of their hosts. Eggs of *A. lumbricoides* have been identified in archeological coprolites in the Americas, Europe, Africa, the Middle East, and New Zealand, the oldest ones being more than 24,000 years old [1]. Humans probably recognized these two ascarids since prehistory, due to their abundance, adult size, symptoms, and distribution [2]. Human ascariasis, with an infection of more than 1.2 billion people, is therefore included in the WHO list of neglected tropical diseases [3, 4]. In pigs, ascariasis leads to significant economic losses, by causing a low feed conversion efficiency and liver condemnation [5].

A. lumbricoides and *A. suum* are closely related to each other [6] and there actually has been a longstanding debate on whether the two parasites are actually a single *Ascaris* species [7-9]. Since *Ascaris* species in both human and pigs are not only almost morphologically and antigenically identical, but also share a similar life cycle and constitute comparable natural host-parasite relationships [10], the *Ascaris*-swine model has been considered as an optimal experimental model to study ascariasis in human at the molecular level [11, 12].

Moreover, very recently, Leles *et al* performed a comprehensive comparison between *A. lumbricoides* and *A. suum* out of an overall review of literatures, and achieved a conclusion that the two parasites belong to a single species, based on the following aspects: 1) Evaluation of mitochondrial markers in several modern parasite samples collected from humans and pigs that kept close contact with the counterpart showed common haplotypes in *Ascaris* spp. derived both from human and pig hosts. 2) There are high levels of genetic similarity between the complete mtDNA genomes of the two *Ascaris* species. 3) In the estimated 10,000 years history of close contact of humans and wild pigs, cross-infections between humans and pigs have been described [13].

In this chapter, an overview will be given of the life cycle, epidemiology and pathology of *Ascaris* spp. infections. Moreover, the current control strategies and prospects of future control strategies will be discussed.

1.1 Life cycle

Among the parasitic helminths, *Ascaris* has one of the most complex life cycles in a single host, involving molts and larval development in several well-defined organ systems (Figure 1.1) [10, 14, 15]. A similar migratory route is observed in human and pig hosts, which is illustrated for the pig in Figure 1.1. Eggs are passed from the host in the one-cell stage. Fully embryonated eggs of *Ascaris* spp. (Figure 1.2A), which contain L3 stage larvae, become infective after two moults. It takes approximately 6-10 weeks for fertile eggs to become infective [16], depending on the environmental conditions (optimum: moist, warm, shaded soil). Hosts become infected *via* the faecal-oral route [17]. After hatching in the gastrointestinal tract covered by the L2 cuticle (Figure 1.2B), the infective L3 larvae penetrate the mucosa of the small intestine at the caecum or proximal colon. The larvae then migrate *via* the mesenteric blood circulation to reach the liver, where the L2 cuticle is shed. Subsequently the larvae reach the lungs 7 days post infection (p.i.) [10, 14]. On 10 days p.i., the larvae enter the alveoles and move up to the trachea where they are swallowed, eventually returning to the small intestine. In total, this hepato-tracheal migration takes place over a two-week period after the uptake of the parasite's eggs by pigs and humans. Throughout the larval migration, the larvae grow continuously, from average length 250 μm at day 0 p.i. to 1,500 μm until they reach the lungs at day 7 p.i., after day 14 p.i. the average length of the fourth stage larvae (Figure 1.2C) are more than 2,000 μm [17]. Worms are mature around 6 weeks p.i. and the adult females (Figure 1.2D) can begin to excrete fertilized eggs (Figure 1.1A). Unembryonated ova enter the environment *via* the faeces and can remain viable in the soil for up to 15 years [18].

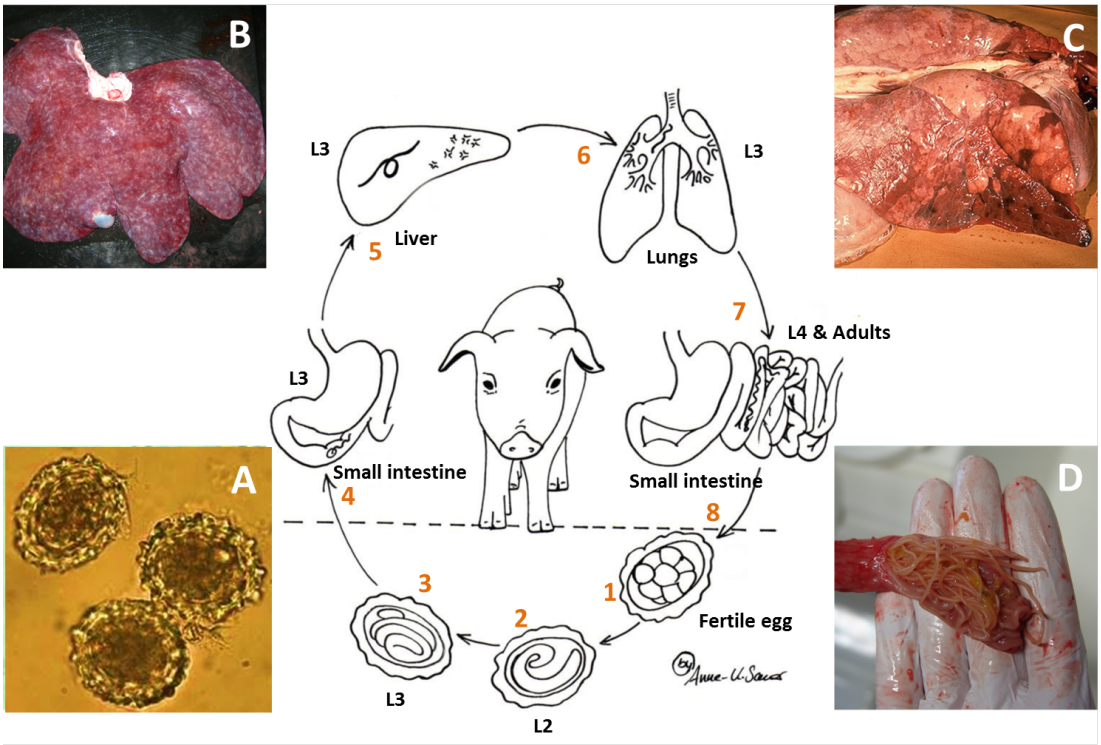

Figure 1.1: Life cycle of *A. suum* in pigs. Fertilized eggs (1) are excreted into the environment with the faeces.After two molts within the eggs, the embryos develop into second stage larvae (2) and third stage larvae (3). After oral intake by host, the third stage larvae hatch in the gastrointestinal tract and penetrate the caecum or colon wall (4). Then, the third stage larvae are transported through the mesenterial blood veins to the liver (5) where they get stuck in the capillaries and destroy liver tissue in order to get to the efferent blood vessels. The blood stream carries the larvae to the next capillary system, which is the lung (6), where they penetrate the alveoli, move up the respiratory tree, and eventually get swallowed again. In the small intestine, the third stage larvae molt to the fourth stage (7). After about 6 weeks, the worms have reached maturity and adult females can begin to excrete fertilized eggs (8). (A) Unembryonated eggs; (B) typical white spot lesions on the liver of a pig killed 14 days after an experimental infection with 500,000 *A. suum* eggs; (C) lung inflammation casued by the migrated larvae; (D) numerous *A. suum* L4 visible inside the small intestine of a pig.

(Based on Anne Sauer; http://www.fsbio-hannover.de/oftheweek/152.htm)

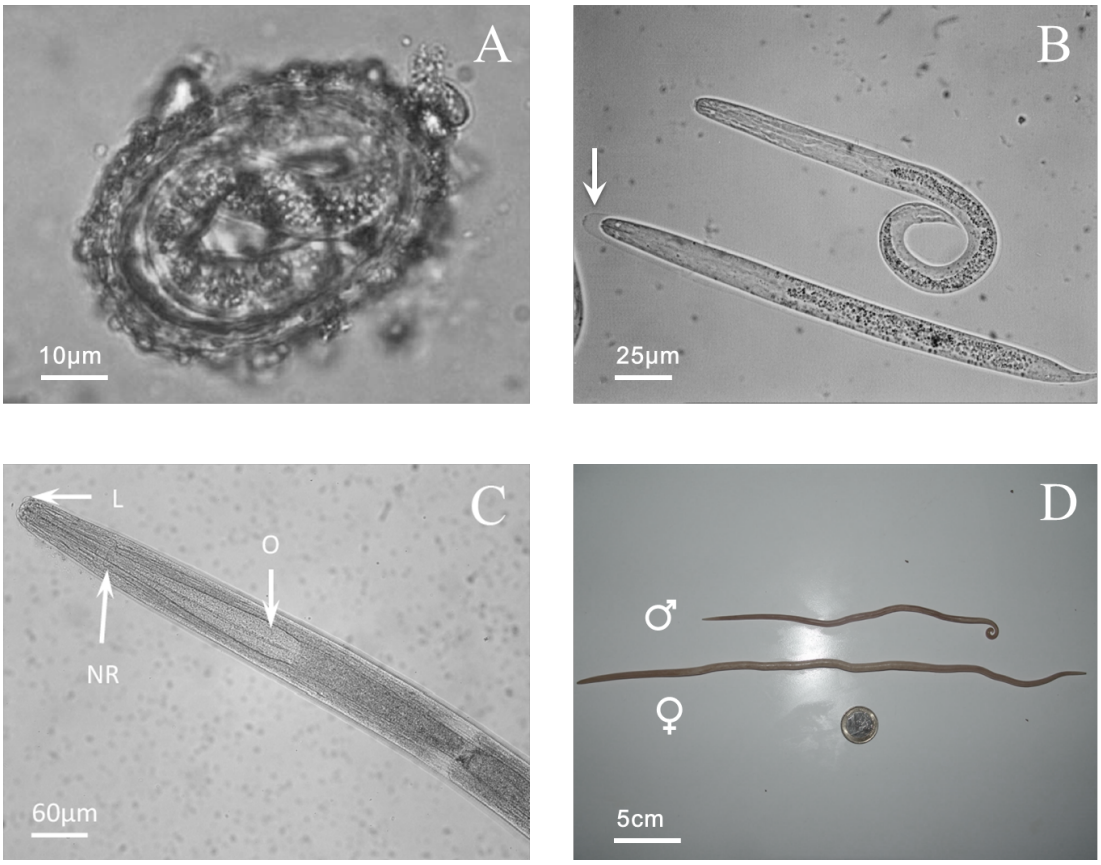

Figure 1.2: Mophology of different stages of *A. suum*. (A) Fully embryonated, infective egg. (B) freshly hatched L3 larvae covered by a visible L2 cuticle (see arrows). (C) Anterior end of L4 larva, recovered from the small intestine at day 14 post-infection. Oesophagus (O), lips (L) and the nerve ring (NR) surrounding the proximal part of the oesophagus is indicated with arrows. (D) Male and female *A. suum* adult worms.
(Based on [19])

1.2 Epidemiology

The distribution of ascariasis prevalence is not only one of the most commonly used measures in occurrence of this health-related disease, but also crucial in the decisions about how to design and manage control programs.

1.2.1 Prevalence of A. lumbricoides infection

In humans, ascariasis is widely distributed throughout the tropics and subtropics. It has been reported in at least 150 countries, particularly within the developing world, such as China, India and sub-Saharan Africa countries [8]. Numerous studies in the 1990s showed that an estimated 1.4 billion people harbor *A. lumbricoides* out of 6 billion world's population. In the first decade of the 21st century, the updated global prevalence has dropped to 1.2 billion [3, 4], which is probably due to China's large-scale 'deworming' programs [20]. The prevalence of *A. lumbricoides* is shown in the world map on Figure 1.3.

Generally, high prevalence of human ascariasis is always linked with poor socio-economic conditions, i.e. defecation practices, geophagia, cultural differences relating to personal and food hygiene, occupational necessity, agricultural factors, housing style, social class and gender [21]. The prevalence/intensity of *A. lumbricoides* is significantly increased in some cities of developing countries due to favorable conditions of transmission, when approximately one third of the population lives in slums and shanty neighborhoods [22]. Worm burden was also observed to be heavier for certain households with more family members [23, 24]. Besides, the infection of human ascariasis is more likely to spread in tropical environments, where wetness and warm temperature acts as an ideal condition for egg survival and embryonation. For instance, in Africa the prevalence is lower in arid climate areas than in warm regions [25].

Notably, the reliability of available data is yet quite limited. Although the reliability of some available data is sometimes questionable, it is reinsuring to see that in some cases independent studies, applying different methods and/or with different levels of access to information, showed similar results [20, 26-28].

However, prevalence measures can still be remarkably different between communities/countries, due to uneven technological and scientific development. For a typical example cited by Crompton [8], the average prevalence of ascariasis in Kenya was considered to be almost 40%, whereas less than 20% prevalence as estimated in Ivory Coast, while both countries are located in the same large region. There also exists data that show variation in prevalence between communities within a single country, for example from 0% to 76% in relatively adjacent villages in Ghana, even with the same methods being applied by the same research group [29].

The intensity of *Ascaris* infections in human does not follow a normal distribution, but exhibits an aggregated or overdispersed frequency distribution, which means that most of the worms aggregated in a very small proportion of hosts, whereas the majority of the other individuals carry few or no worms [30-32]. The hosts harboring disproportionately large worm burdens have a higher risk of morbidity and mortality, and also act as significant contributors of potentially infective stages in the environment. In this epidemiological pattern, heavily infected individuals, referred to as 'wormy persons' [30], are highly conditioned by age and sex [31, 32]. The most intense *A. lumbricoides* infections often occur in children aged 5–15 years, with a decline in intensity and frequency in adults. Such age-dependent intensity variation may suggest the antigen exposure and/or acquired immune response in the hosts. The principles of overdispersion and predisposition are still not fully clear. It was supposed that the host genetics and host socio-economic status contribute nearly as much as host acquired immune response to the observed heterogeneity of infection intensity [33]. However, Chan *et al*. [34] noted that environmental or behavioral features of the family household are major determinants of parasitic infection status.

1.2.2 Prevalence of A. suum infection

In pigs, ascariasis is presumably present in pig farms all over the world. In contrast to well-documented infection rates of *A. lumbricoides* in humans, only 19 countries have up-to-date information of the prevalence of *A. suum* in pigs

[35], which are shown in the world map (Figure 1.4). Besides, the presence-absence situation of *Ascaris* in different pig farms was determined by different investigators, usually in different manners. For instance, some research groups prefer to use the percentage of rejected livers as a measure for parasite exposure, while others would rather check the intestinal tract at slaughter or examine faecal samples for parasite eggs to assess the worm loads. Hence, it is not possible to draw a global conclusion on epidemiological trends of ascariasis on pig farms. Still, according to the limited but valuable reports on prevalence of *A. suum* infection, it has been shown that pig ascariasis is prevalent on quite a number of pig farms across the world, and that the situation has not changed significantly over the last few decades. For example, the pig ascariasis distribution of Denmark in 1989 and 2010 stayed roughly at the same level, with *A. suum* being present on 88% and 76% of all investigated farms, as shown by two comparable studies [36, 37].

Like the overdispersion of *A. lumbricoides*, the prevalence of *A. suum* infections differs among sows, boars and fatteners, with the highest prevalence in breeding sows and fatteners [38]. This was further confirmed by several studies in Denmark and China [39-41]. In some extreme cases, worm eggs presented in the farrowing pens of intensive herds were abundant, while excreted eggs were hardly found in pens for weaners [39, 41-43]. Under indoor conditions, piglets could easily get massive *A. suum* infection after transferring from farrowing stys to highly infective fattening units [38, 41, 44].

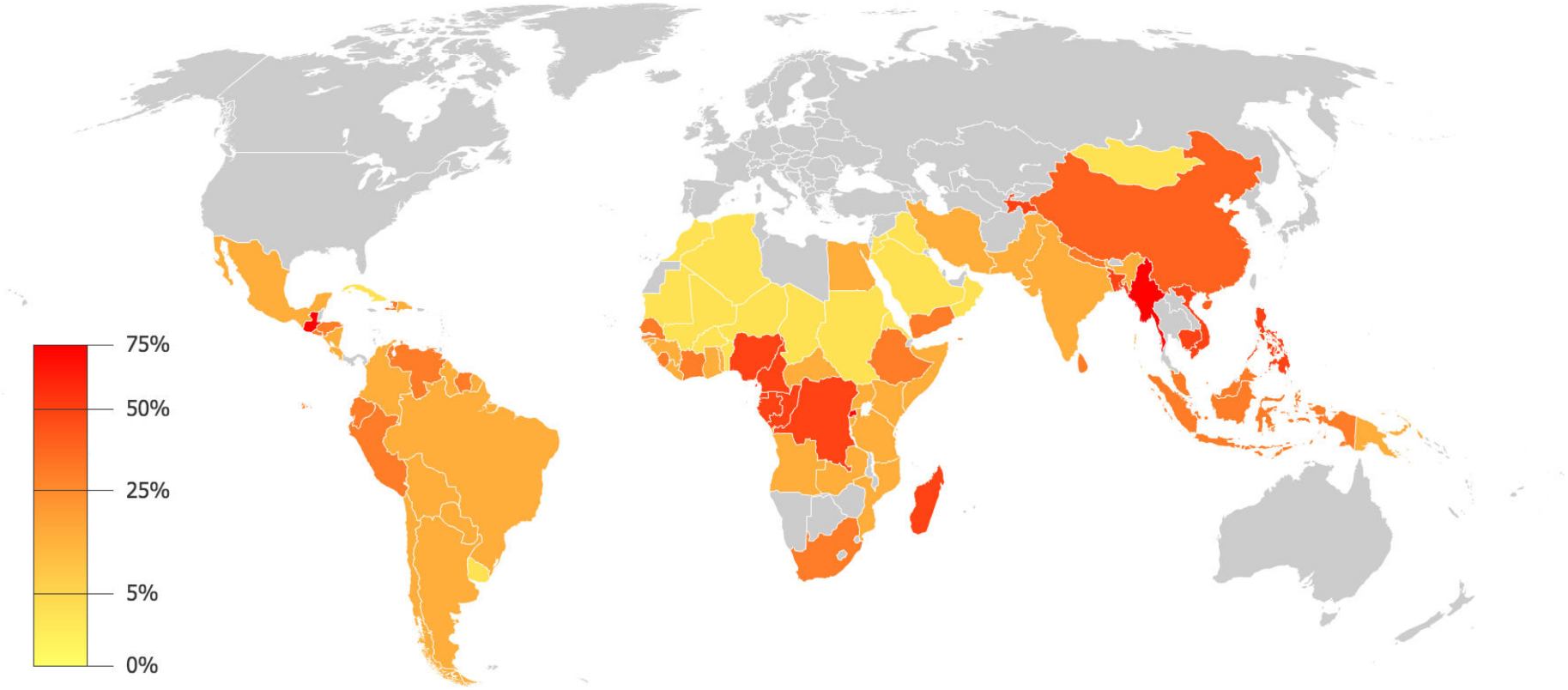

Figure 1.3: The global distribution of *Ascaris lumbricoides* infections. Countries with the prevalence data of *A. lumbricoides* infections in the analysis were highlighted. The different color show different the prevalence rate, which are indicated on the left bar. Grey areas represent the countries that haven't been analyzed for the prevalence of human ascariasis at the present.
(Based on [3])

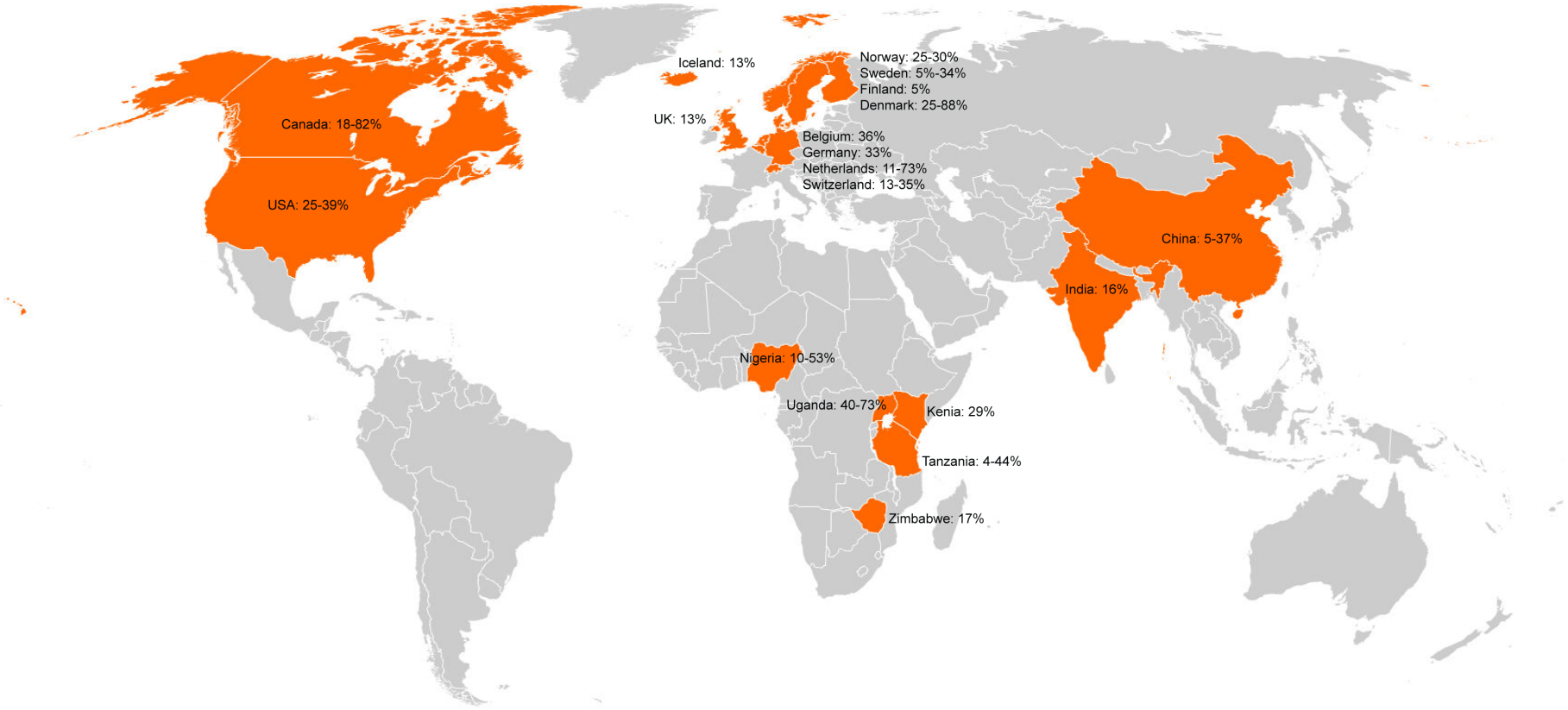

Figure 1.4: The countries with available prevalence information of *Ascaris suum* in pigs. Nineteen countries included in the analysis were highlighted (in orange color), marked along with the latest prevalence data of *A. suum* infections in pigs. Grey areas represent the countries that haven't been analyzed for the prevalence of pig ascariasis at the present.
(Based on [35])

1.3 Pathology & Economic loss

1.3.1 A. lumbricoides

Since the morbidity and mortality of ascariasis are directly related to the worm load, those who harbor light infections tend to be asymptomatic. An estimated 8-15% (120-220 million cases) of *A. lumbricoides* infected patients demonstrate associated morbidity [45, 46]. Only few individuals harbor sufficient worms to precipitate life threatening [47].

Since *Ascaris* infection causes more disability than death, the worldwide burden of ascariasis, as for many neglected tropical diseases, is typically assessed by disability-adjusted life years (DALYs). DALY is a measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death. There is an estimated 10.5 million DALYs lost associated with *A. lumbricoides* infection, while the combined DALYs for the soil-transmitted helminthes (STHs), *A. lumbricoides*, *Trichuris trichiura* and hookworms are 39.0 million, which is higher than the DALYs estimated for malaria (35.7 million) [45]. The globe distribution of DALYs for ascariasis per 100,000 inhabitants is shown in Figure 1.5.

Most of the clinical features of human ascariasis are (in)directly caused by larval migration and adult gastrointestinal parasitism. The clinical features and general outcomes associated with *Ascaris* infection are summarized in Table 1.1. Human hosts tend to suffer from verminous pneumonia, asthma, dyspnea, cough and fever because of larval migration through the pulmonary tissue, whereas abdominal distension and pain, nausea, diarrhea and appetite loss are clinical symptoms resulting from adult worm gastrointestinal parasitism [8]. In some extreme cases, the entangled adult worms can lead to mechanical intestinal obstruction, which can be life threatening [47]. For children particularly, *Ascaris* infection is demonstrated to cause a chronic influence on host nutrition [48], with infected children showing growth and cognitive impairment [48, 49].

1.3.2 A. suum

A. suum infections rarely cause specific clinical symptoms in pigs [50, 51], except that the migration of larvae through lungs could cause very few cases of acute symptoms, such as coughing and wheezing (Table 1.1). However, *Ascaris* infections in pigs do appear to have a major economic impact on the pig industry rather than great impact on the pig's health [52, 53].

When the *A. suum* larvae migrate through the liver tissue of their host, significant tissue damage is caused, which appears as "white spots" on the superficial hepatic surface and within the liver tissue of infected pigs. These white spots are the most representative pathological lesions caused by migrating *A. suum* larvae in pigs, normally formed by the mechanical injury and inflammatory response induced by the migrating larvae [54]. In total, there are three types of white spots in *A. suum* infected pigs, including the compact and mesh-worked white spots, both of which are produced by eosinophilic interstitial hepatitis, and the lymphonodular type of spots formed by lymphofollicular hyperplasia [55, 56]. White spots can already be observed 3 days p.i. and start to resolve after about 2-3 weeks p.i. [40, 57]. Depending on the numbers of white spots, the contaminated livers are trimmed or fully rejected at slaughter, resulting in obvious economic losses.

When larvae migrate through lungs, not only direct physical damage is caused to the lung, but also the development of pneumonia or pleuritis is subsequently promoted. Importantly, many studies showed that migrating *A. suum* larvae in the lungs may exacerbate lung infections caused by other pathogens, such as *Pasteurella multocida*, *Escherichia coli* and *Salmonella* spp. [58-61]. In addition, *A. suum* infections were observed to have a significant negative effect on the seroconversion and antibody levels to a *Mycoplasma hyopneumoniae* vaccine [62]. Both the negative effects on the productivity caused by bacterial or viral infections and the costs of the treatment for these diseases contribute to the economic impact of ascariasis.

Moreover, parasitism of adult *A. suum* in the intestine of pigs is also known to be responsible for villus atrophy, impaired absorption of vitamin A and temporary

lactose intolerance [12, 56, 63]. Consequently, the infected pigs show decreased daily weight gain and feed conversion efficiency, which of course result in considerable economic loss.

Like most diseases caused by parasites, due to non-lethalness and numerous interaction factors, estimating the economic impact of *A. suum* infection on pig performance is not a straightforward task. In 2001, ascariasis caused an estimated US$ 77.6 million loss to pig producers in the US, 22.6% (US$ 17.5 million) is because of liver condemnation and the rest 77.4% (US$ 60.1 million) due to a higher feed conversion rate [64]. More recently, Van Meensel *et al*. [65] estimated that the losses of *A. suum* infections are up to € 4.61 per pig. Hence, strategic deworming offers a win-win effect on both economic and environmental performances with gross profit margin increases of 3 to 12 euro per pig per year (reviewed in [35]).

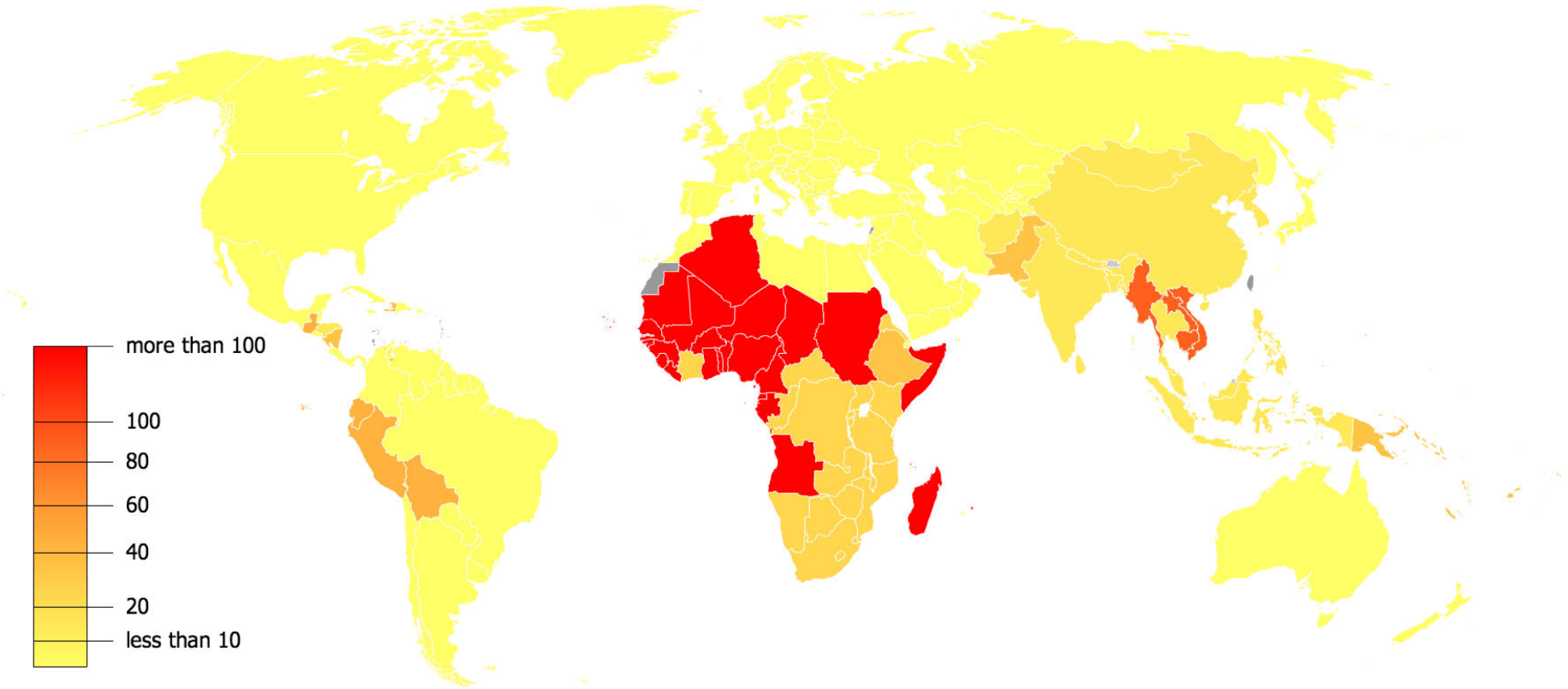

Figure 1.5: The globe distribution of DALYs for ascariasis per 100,000 inhabitants. The different color showed different the DALYs values, which indicated on the left bar. Grey areas represent countries not included in the present analysis.
(Based on [66])

Table 1.1: Clinical features and general outcomes associated with *A. lumbricoides* and *A. suum* infection

Species	Stage	Clinical features/syndromes	General outcomes
A. lumbricoides	Larval migration (through liver and lungs)	Verminous pneumonia, asthma, dyspnea, cough, substernal pain and fever	Impaired growth
	Adult gastrointestinal parasitism	Abdominal distension and pain, nausea and diarrhea, appetite loss, lactose maldigestion, intestinal obstruction, intussusception, volvulus	Impaired physical fitness Impaired cognition Reduction in school attendance and performance
A. suum	Larval migration (through liver and lungs)	Hepatic white spots, coughing, wheezing, loss of appetite and depression	Condemnation of livers at slaughter Impaired growth
	Adult gastrointestinal parasitism	Malnourish, reduced food intake and growth rate, impaired lactase activity in the intestinal mucosa	Decreased daily gain and feed conversion efficiency

(Based on [8, 35, 67])

1.4 Immunity development against infections with Ascaris

In general, the immune response of human/pigs to *Ascaris* infection, like that of all other vertebrates, can be divided into innate resistance and adaptive immunity. Evidence shows that the *Ascaris* worm load is predominantly regulated by the genetic constitution of the host [68-70]. The quantitative genetic studies of pedigrees from Nepalese and Chinese communities have illustrated a substantial genetic component that could determine host variation in susceptibility to *A. lumbricoides* (30–50%) [68]. For *A. suum*, the genetic contribution (heritability) was believed to account for 30–70% of the variation in worm burdens [69].

Another important host defense against infectious diseases is antigen-specific acquired immunity. The immune responses against *Ascaris* have been under investigation for decades. *Ascaris* is generally considered highly immunogenic in both humans and pigs [71, 72]. Like other helminths, it has been described that *Ascaris* can evoke strong Th2 skewed responses, which can be measured both systemically (e.g. blood eosinophilia, IL4) and locally (increased IL4, IL6, IL10, and IL13) [73, 74]. The cell-mediated immune responses against the parasite are described to play an essential role in the resistance of *Ascaris* and in the development of resistance to re-infection [71, 72].

During a primary infection, the migrating L3s cause pathological lesions in the gut, liver and lungs. Consequently, a short-lived immunological reaction against the larvae, which is characterized by the production of B cells and $CD4^+$ T cells in the local lymph nodes, is observed in the liver by day 7 p.i. [75]. Two weeks after the infection, the hepatic immunological reaction evolves into pulmonary immune response, with enlarged lung-associated lymph nodes [75].

Investigations demonstrated that, up to 50% of the administered infective *A. suum* larvae could survive until day 14 p.i. and reach the L4 stage [43], however, between day 14 and day 21 p.i., there were more than 95% of the L4 larvae being gradually eliminated from the small intestine. This is known as the self-cure

reaction or expulsion phase [40, 76]. It is possible that the expulsion of *Ascaris* worms results from a density-dependent self-reduction of the larval population. Nevertheless, an intestinal hypersensitivity reaction was seen after the hepato-tracheal migration, characterized by an accumulation of mast cells, eosinophils and IgA-producing cells in the gut mucosa. A hypothesis that a specific immune-mediated reaction, which is mainly induced by Th2-derived cytokines, helps to prevent the larvae returning to the intestine is widely accepted [72, 77-79].

After repeated exposure to infective eggs, a second line of defense in addition to the non-specific immune system develops at the level of the intestine that stops new incoming *Ascaris* larvae from successfully penetrating the intestinal wall to reach the liver [51, 80]. This specific immunological process is called the pre-hepatic barrier [71, 80-82], and the location of the protective mechanism was demonstrated to be at the level of the gut, because injecting *in vitro* hatched L3 larvae into the mesenteric veins caused white spots formation, while orally administering eggs did not [71]. However, the functionality of the pre-hepatic barrier is still not fully understood and requires further investigation [71, 81, 82]. It is presumed that a close interplay between immunological and histophysiological aspects, such as tunica muscularis hypertrophy and surface mucosa alteration [11] may be essential in the protective intestinal immunity. As a local response to the migratory larvae, intensive infiltrations of eosinophils were found in histopathological sections of lung tissue in infected mice and pigs [57, 83, 84]. Frontera (2004) proposed that eosinophils seem to be associated with protection within pigs that had been repeatedly infected or immunized with worm antigens [84], and the crucial role of eosinophils in generating pre-hepatic immune barrier has been proven very recently [85].

1.5 Control strategy

1.5.1 Control strategies of human ascariasis in practice

Sanitary measures, health education and large-scale anthelminthic treatment (chemotherapy) are considered as three major strategies for control of human ascariasis [86]. Sanitation-based control programs aim to interrupt transmission

and prevent re-infection. In this way, worm loads can be gradually reduced [87]. Furthermore, health education could encourage sanitation and stimulate changes in environmental and family hygiene-related behaviors. However, despite the well-recognized role of effective sanitation and health education in preventing transmission of intestinal helminths, elimination of *A. lumbricoides* infection is hardly achieved in a short period of time, owing to the scarcity of resources in most developing countries. To accomplish the short-term goal of reducing infection load and transmission potential, in order to bring down morbidity to a more tolerable level and prevent mortality associated with the disease in a very near future, the current helminth control strategies are focused on developing proper cost-effective approaches, employing available chemotherapeutic agents [88].

Nevertheless, unorganized treatment of individual cases does not contribute much to the control of ascariasis in communities. Three efficient treatment strategies, universal, targeted or selective anthelminthic chemotherapy, are highly recommended in community control programs [46, 89]. Considering that *A. lumbricoides* is commonly co-endemic with two other STHs species, i.e. *T. trichiura* (whipworm), and *Ancylostoma duodenale*/*Necator americanus* (hookworms), the chemotherapy is usually targeted at all three STHs infections together. Besides, designs for chemotherapy programs should be based on recent and reliable information of epidemiologic conditions, the target people, seasonality, the associated morbidity, and the re-infection rate [46]. In general, (1) universal drug administration is offered to all individuals irrespective of the age, gender, worm burden or other social characteristics in areas with a STH prevalence higher than 20% [4]. (2) targeted drug administration is offered to a group within a community where groups may be defined by age, gender, religion or other social characteristics, such as school children or pre-school children. Targeted treatment once every year is advised by WHO for school-age children with a prevalence between 50 and 70%, and twice/ thrice per year for that with a prevalence more than 70% [90]. (3) Selective drug administration, according to intensity of current or past infection, is offered only when adequate laboratory facilities are available. In practice, a series of studies in Nigerian villages have

been performed to compare the efficacy of universal, targeted and selective treatment strategies with levamisole. Results showed that both universal and targeted treatment were the most effective approaches in a high risk group of school children and having an effect on reducing the *Ascaris* infection risk in untreated adults. [87].

For the selection of anthelmintics in helminth infections control strategies, there are several essentials to consider, namely (1) the safety of the used compounds, (2) the efficacy of the drugs, (3) the spectrum of activity, (4) the local drug administration rules, and (5) the price of the chosen anthelmintic drugs. So far, four anthelminthic drugs, Albendazole, Levamisole, Mebendazole and Pyrantel embonate, have been proven to be highly effective against *A. lumbricoides*, with over 90% cure rate and an estimated cost of $0.02-0.03 per individual. According to the most updated WHO guidelines for the control of STHs, the two benzimidazole drugs, i.e. albendazole and mebendazole, are predominantly used in practice for chemotherapeutic control programs of human ascariasis and morbidity associated with other STHs [91].

1.5.2 Control strategies of pig ascariasis in practice

Transmission of *A. suum* among pig populations is dependent on factors such as housing system, hygiene, management practices and anthelminthic treatment [92]. Therefore, similar to the control of human ascariasis, effective control of *A. suum* infections on pig farms relies not only on mass antiparasitic treatment, but also on good general farm management and increased hygienic standards [38, 93].

There are numerous aspects of farm management that can have a major impact on the epidemiology of *A. suum*, including (1) the practical pig stocking density for indoor systems, (2) feces disposal, (3) water supply, (4) production system, and last but not least (5) sty cleaning protocols. (1) Logically, as the stocking density rises in the pig farm, it becomes more likely to detect *A. suum* infected pigs in the stys. Hence, it is important to limit the density to a proper level [94]. (2) It is important to reduce the contact of pigs with faecal deposits of their own

or of the pigs of former distinct groups. Previous studies indicated that housing of pigs on slatted floors was associated with a decreased prevalence of parasite infection compared to solid or partially slatted floors, owing to the separation of the animals from *A. suum* eggs in faeces [93-95]. Notably, with increasing animal welfare concerns, more and more bedding materials are being used in the stys nowadays. This actually provides extra breeding ground for parasite eggs embryonation [35]. (3) Moving the water supply system away from the lying area and the feeding troughs can avoid a constant humidity environment in these areas, and therefore lower the survival chances of the parasite eggs. (4) It is important to protect the farms as much as possible from exterior *A. suum*, which could soon spread all over a farm, *via* incoming animals, dirty materials and boots, and even flies [96]. Recent studies showed that the risk of introducing new infections into the herd is positively correlated with importing pigs from piglet producers. In intensive indoor systems, the farms that have their own breeding stocks tend to harbor lower *A. suum* burden. For fattening farms, it is preferred to import piglets from larger piglet producers with good management and hygiene [42]. Necessarily, incoming pigs should be treated prior to delivery or/and upon arrival, to obtain worm-free status and reduce the risk of introducing the infection into their herds. In addition, all-in-all-out production, which means pigs are moved into and out of facilities in distinct groups, is a good system to avoid the commingling of pig groups. This practice also provides an occasion to clean and disinfect the farm thoroughly between subsequent groups of animals. The all-in-all-out system is widely used in the hope of decreasing the spread of disease, and has been linked to a lower prevalence of *A. suum* [42, 93, 94]. (5) Regular and frequent cleaning of the stys is required, at least after each fattening cycle. However, the method applied is of importance. A thoroughgoing cleaning with high-pressure water could remove most of the residual faeces, which is helpful in reducing the amount of eggs in the environment, yet not effective enough to fully eliminate all of the infective eggs [38]. On the other hand, steam cleaning and drying of the stys can destroy especially roundworm eggs. But this method is quite difficult to perform. Under laboratory conditions, the disinfectant Neopredisan (active principle: p-chlorine-m-cresol) showed a reliable (100%) efficacy against *A. suum* eggs [97].

Anthelmintics such as dichlorvos, piperazine, pyrantel, fenbendazole, flubendazole, oxibendazole, ivermectin, doramectin and levamisole have all been proven as highly effective (92.4%-100%) products against adult roundworms in pigs (Table 1.2) [98-108]. Recommended doses of pyrantel, fenbendazole, flubendazole or levamisole also showed antiparasitic activity against migrating stages of *A. suum* [102, 104, 105, 109]. Yet, many of those anthelmintic products are not commercially available, only the benzimidazoles and levamisole are actually the most frequently used products against *Ascaris* in the pig industry.

Table 1.2: Efficacy of different anthelminthic drugs tested against *A. lumbricoides* and *A. suum*

Infection	Anthelmintic drug	Formulation	Dose	Effective rate	Reference
A. lumbricoides	Albendazole	Orally	400 mg once	>90%	[8, 67]
	Levamisole	Orally	2.5 mg/kg once	>90%	[8, 67]
	Mebendazole	Orally	500 mg once	>90%	[8, 67]
	Pyrantel embonate	Orally	11 mg/kg for 3 days	>90%	[8, 67]
A. suum	Dichlorvos	Orally	43 mg/kg once	100%	[107]
	Doramectin	Injection	300 µg/kg once	100%	[108, 110-112]
	Fenbendazole	In feed	3 mg/kg for 3 days	92.4-100%	[98, 100, 113]
	Flubendazole	In feed	5 mg/kg once	100%	[101]
	Levamisole	In feed	8 mg/kg once	100%	[104]
	Ivermectin	In feed	2 ppm for 7 days	100%	[106]
	Oxibendazole	Orally	15 mg/kg once	100%	[103]
	Piperazine	In water	200 mg/kg once	100%	[99]
	Pyrantel Tartrate	In feed	96 g/ton feed for 24 days	100%	[105]

(Based on [8, 35, 67])

1.5.3 Problems and limitations in the current control strategies against ascariasis

At this moment, the use of chemical drugs to treat *Ascaris* spp. infection still accounts for most of the current control strategies in practice. However, despite the high efficacy of the available products, their activity is only limited to a couple of days following treatment. This results in a rapid reinfection of the host, especially since female *Ascaris* worms are extremely fecund and can produce hundreds of thousands of eggs per day [8], leaving a highly contaminated environment. In the countries where sanitation and hygienic conditions are insufficient, it was shown that within 11 months after community-wide treatment, the rate of *A. lumbricoides* infection reached 55% of pretreatment rates [114]. The same situation can be found in the pig farms.

In response to the reinfections, anthelmintic drugs are repeatedly administered, normally at short time intervals. However, as mentioned before (section 1.5.1 and 1.5.2), the benzimidazoles are the only anthelmintics actually used against *Ascaris* infection in human. All benzimidazole-based compounds act through the same therapeutic mechanism, i.e. binding with high affinity and in a pseudo-irreversible fashion to the beta-subunit of the tubulin protein, disrupting microtubules structure and functions of nematode [115]. Therefore, as a side-effect, the more intensively these chemical compounds are being used in treatment for parasites, the more likely anthelmintic resistance will develop [116]. Even more unfortunately, the resistance to drugs is hereditary [117, 118], and can be passed on from the exceptionally surviving worms to their offspring [119]. Furthermore, selection pressure through successive generations on the arisen resistance alleles is accelerated by frequent treatments with the same anthelmintic in the parasite population. Consequently, at a certain time point, the drug could no longer threaten the parasites.

In recent years, multidrug-resistance has considerably emerged across five continents in the strongylate nematodes of domesticated animals [118, 120]. Appearance of severe drug-resistance even has stopped sheep and goat farming in some areas of South Africa, New Zealand, Australia and the UK [121-123]. So far, there has no report sugguesting the exist of resistant *A. lumbricoides* and/or

A. suum [118, 124]. Nevertheless, theoretical studies, with the support of the evidence of pesticide resistance development, indicate that it is only a matter of time for drug resistant *Ascaris* spp. to appear eventually [125, 126]. To continue protecting the host from parasite infections, new control tools, such as other drugs with a different mode of action or vaccination, are needed.

1.5.4 Alternative control strategy of ascariasis – vaccination

Concerns about the sustainability of periodic treatment with benzimidazoles and the foreseeable emergence of drug resistance have prompted constant efforts to develop and test new control tools. Vaccination is believed to be the method of choice for the control of ascariasis, since it provides the possibility of a safe, environmentally friendly, durable protective and potentially cost-effective control strategy against *Ascaris* infection. In addition, given that the vaccine targets are normally different from those of anthelmintic drugs, vaccination could be synergistic with anthelmintics. Ideally, combining vaccines with drugs should be able to reduce parasite infection intensities, morbidity, and transmission within a favorable short period, and at the meantime induce constant protective host immune responses, precluding the risk of both drug failure and reinfection. This would be an inspiring novel concept of control activities against ascariasis, integrating the short-term effects with long-term elimination goals [127].

For about half a century, considerable attention and research effort has been directed towards the development of a vaccine against *Ascaris*, either based upon naturally exposed or hidden antigens. It was very encouraging to find out that hosts can build up significant acquired resistance after repeated exposure to infectious eggs or L3s of *A. suum*, as reviewed in section 1.4 [76, 128], suggesting that vaccination against this parasite is feasible.

Theoretically, there are several types of anti-nematode vaccines that can be used, including vaccines based on live attenuated material, whole material of dead organisms, (semi-) purified native antigens and recombinant antigens [129]. An overview of the different vaccination trials previously performed against *Ascaris*

is given in Table 1.3. Earlier experiments were mainly focused on immunization of pigs with UV-irradiated eggs or worms. Tromba (1978) challenged pigs with 500 UV-irradiated *A. suum* eggs for 3 times, and found out that the number of adult worms recovered from animals of the treated group reduced by 86%, compared with that from the control pigs [130]. Within the next few years, the same team further tried to immunize pigs with inactivated *A. suum* eggs at different dose levels for different times of inoculation and in different administration ways. At highest, a level of 94% protection was induced by immunization with 5 oral inoculations of 10,000 UV-irradiated eggs at total energy doses of 75-150 mu W-min/cm^2 [131-133].

Aside from inactivated eggs, many other body materials of *A. suum*, such as body wall, hatching fluid and excretory-secretory (ES) products of L3/L4 larvae were also tested for immunization of animals, yet less successful [134, 135]. In pigs, a combined immunization with both egg and larval products raised significant protective immunity to a challenge exposure with 10,000 *A. suum* eggs, but with a marked pathological response to larvae migrating in the liver [131]. On the other hand, embedding 100 mg of antigen in a liposome with an immunomodulator (levamisole) succeeded in inducing 89% reduction in lung and liver larvae retrieved from mice [136]. Except for raw worm materials, some purified native antigens were also examined for their protective capacity, in only one study though [137]. Two out of three tested antigen molecules (14 and 42 kDa) resulted in significant levels of immune protection against lung stage larvae (67-93%) in pigs after 6 times of weekly inoculations of 1μg of antigen in Freund's incomplete adjuvant [137].

In addition to native parasite products, several recombinantly produced proteins have also been evaluated. The first vaccination trial of protective recombinant antigen (rAs14) against *A. suum* infection in laboratory animals was reported by Tsuji and colleague in 2001[138]. Intranasally administration of rAs14 coupled to cholera toxin subunit B to mice for three times resulted in 64% reduction of the larvae recovered from the lungs, in comparison with the non-treated controls. In this process, the total serum IgG levels, the mucosal IgA responses,

and the rAs14-specific IgE response in the vaccinated animals were significantly increased. Deeper investigation into the IgG subclasses in the sera of the immunized mice showed that IgG1 level was highly elevated, but the level of IgG2a antibody response was brought down, which suggested that the specific protective immune response was associated with a type II immune reaction. In pigs, after immunization with another recombinant antigen (rAs16), a 58% reduction in the recovery of lung-stage L3 larvae was observed that was associated with increased levels of IL-4 and IL-10 and high titers of rAs16-specific mucosal IgA and serum IgG antibody, indicating that rAs-16 was capable of interfering with larval survival through type II immune response just like rAs14 [139]. Similarly, subcutaneously injecting recombinant As24 in Freund's complete adjuvant into laboratory mice successfully yielded 58% of immune protection [140], whereas no protection was generated when mice were immunized with As37 recombinant antigen in a comparable setup [141]. Most recently, enolase was also validated as a potential vaccine candidate against ascariasis, inducing a 61% decrease in recovered larvae in mice [142].

For helminths, there exist many practical problems in applying vaccines based on native material. One of the most critical issues is that it is very difficult to acquire large quantities of worm material or native antigens from most helminth species. In this regard, a suitable alternative to native material is demanded. Along with the development of molecular biology techniques, the opted vaccine antigens in recent studies on alternative ways to control *Ascaris* parasites have shifted gradually from native parasite products to recombinantly produced proteins. For the evaluated *Ascaris* recombinant vaccine candidates above, the highest protection rate that could be achieved in vaccination is only 60%, which is still too low to meet the commercialization requirement. On one hand, the low efficacy in exploration of recombinant *Ascaris* vaccine may be caused by poorly designed expression systems for producing recombinant proteins. On the other hand, when researchers noticed that 4 out of 5 antigens (As14, 16, 24, 37) were selected on the basis of immune recognition using sera from animals immune to the worms and then blotted to the antigens and subsequently cloned for recombinant expression, they may have overestimated the importance of

immunological markers such as parasite-specific antibodies in anticipating vaccine efficacy. In the later investigations, one should bear in mind that the immune recognition *in vitro* is not a guarantee of protection *in vivo*, because it has been demonstrated that many parasites can manipulate the host immune responses to their favor [143].

Table 1.3: Vaccination studies performed against *ascariasis*

Year	Antigen	Immunization protocol	Host	Recovered stage	% Reduction	Reference
1978	UV-eggs	3 challenges with 500 UV-eggs	Pigs	Adults	86%	[144]
1982	UV-eggs	10,000 UV- eggs orally 3 successive weeks,	Pigs	Lung larvae	88%	[145]
1984	UV-eggs	2-5 oral inoculations of 100 - 10,000 UV-eggs	Pigs	Lung larvae	83-94%	[133]
1985	Hatching fluid L3 ES L3/4 ES UV-eggs	A number of immunization experiments using different antigens + Alum w/wo oral administration of UV-eggs.	Pigs	Lung larvae	Max 80%	[131]
1988	L3 larval body wall	Oral administration of antigen (0.2-11mg) in liposome complex	Pigs	Lung Larvae	N.S.	[135]
1992	Adult crude antigen	2 doses of 100 mg antigen in liposome, w/wo immunomodulator: levamisole	Mice	Lung and liver larvae together	89%	[136]
1994	L3 cuticle Ag adult cuticle Ag UV-eggs Dirt lot	3 immunizations with 300µg antigen/shot + FIA or 1x 10,000 UV-eggs.	Pigs	Lung larvae	49% 44% 89% 98%	[134]
2001	As14 recombinant	3 intranasal immunizations with rAs14 coupled to CT-B	Mice	Lung larvae	64%	[138]
2002	As37 recombinant	3 immunizations with rAs37 in FCA	Mice	Lung larvae	N.S.	[141]
2001	Eggs Eggs and pyrantel 14kDa 42kDa 97kDa	6 weekly immunizations 1µg antigen in FIA	Pigs	Lung larvae	99% 99% 88% 77% 50%	[137]
2003	As16 recombinant	3 intranasal immunizations with rAs16 coupled to CT-B	Mice	Lung larvae	58%	[146]
2004	As16 recombinant	3 intranasal immunizations with rAs16 coupled to CT-B.	Pigs	Lung larvae	58%	[139]

2005	As24 recombinant	3 immunizations with rAs24 in FCA	Mice	Lung larvae	58%	[140]
2008	As16 recombinant	As16 fused to CT-B expressed in rice and fed to mice	Mice	Lung larvae	Not mentioned but significant.	[147]
2011	Native *A. suum* Haemoglobin (AsHb)	3 Immunizations with AsHb + QuilA	Pigs	L4, L5 and Adults	N.S.	[148]
2012	Recombinant *A. suum* enolase	3 immunizations with 100 µg pVAX-Enol DNA	Mice	Lung larvae	61%	[149]

UV-eggs, eggs attenuated by UV-radiation; N.S., not significant; w/wo, with or without; CT-B, Cholera Toxin subunit B; FCA, Freund's Complete Adjuvant; FIA, Freund's Incomplete Adjuvant.
(Based on [35])

1.6 Concluding thoughts

There is no doubt that, as one of the most prevalent helminthic diseases, ascariasis causes serious public health problems and significant economic loss in pig industry. As long as poverty persists in developing countries, *Ascaris* infections will remain a worldwide health threat and pig industry's enemies. Despite that the modern pharmacological approaches are still effective for the treatment of ascariasis, the widespreadness and persistence of *Ascaris* spp. make it yet difficult to be eradicated in a long-term perspective. In addition, the development of anthelmintic resistance which has been observed in other nematodes, suggests that current repeated doses of massive chemotherapy treatment will probably also lead to drug resistance in *Ascaris* spp. eventually. Therefore, investigation of the alternative means of ascariasis control such as vaccination is worthwhile for pursuing.

To promote the rational development of an effective vaccine, it would be necessary to gain knowledge on molecules which play physically essential roles in survival, which navigate parasite migration to complete their life cycles, which allow parasite to evade the host immune system, and which protect the parasite from the defense response of the host. To discover those key molecules, i.e. potential antigen, a better understanding of the molecular biology and host-parasite relationships of *Ascaris* is required. For example, the development of genome sequencing technology could help to identify vaccine targets with interesting genetic patterns that contribute to stronger vaccine response. The latest application of recombinant DNA techniques for cloning of parasite receptors, ion channels and proteins involved in signal transduction is also believed to result in a rapid progress in recombinant vaccine production. Thus, a solid foundation of knowledge on genomic/transcriptomic/proteomic identification of parasite-derived molecules involved in vital processes, on host-parasite interaction processes, and on the mechanism of how *Ascaris* develops and survives within the host would lead to the determination of novel candidate vaccine-targets. Subsequently, all the information could lead to a more efficient vaccine development, with these better and specific targets to elicit more

successful protective immune responses. Hereby, this thesis will mainly study the basic molecular biology and identify the key players in host-parasite interaction processes based on the *Ascaris*-swine model.

Objectives

Objectives

As described in the general introduction (Chapter one), *A. suum* not just causes a significant economic loss in pig industry, but also is the ideal experimental model to study ascariasis in humans. However, limited information on the basic biology of *A. suum* is available, either has the natural process of its host-parasite interaction been elaborated. Due to the self-cure reaction in the host, very few parasites could survive to adulthood, only less than 5% host have to experience the infection of *A. suum* adults. Nevertheless, all the infected hosts have been exposed to larvae stages *A. suum*. From this point of view, it is more meaningful and practical to develop ascariasis control strategies against larvae other than adult worms. Besides, preventing and curing ascariasis at an early stage could minimize the harm caused to the patients by larval migration. Moreover, the larvae undergo hepato-tracheal migration in the livers, lungs, and the blood, where immune reactions are normally induced strongly. Therefore, the larval stages are suitable and of great importance to investigate for developing effective control strategies. For the purpose of providing fundamental information to support the concerted efforts on the development of novel control strategies, such as vaccination, the overall objective of this study is to investigate the basic biology of *A. suum* larvae and to identify molecules which play essential roles in parasite survival and development during the hepato-tracheal migration. The specific aims of this project were:

1. To explore and analyze *A. suum* larval transcriptomes (Chapter two).
2. To investigate the excretory-secretory products from larval stages of *A. suum* (Chapter three).
3. To identify the surface exposed proteins of the infective stage larvae of *A. suum* (Chapter four).

CHAPTER TWO

Transcriptomic analysis of *Ascaris suum* larvae during their hepato-tracheal migration

Based on:

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2.1 Introduction

As mentioned in chapter 1, section 1.1, *Ascaris* has one of the most complex life cycles in a single host. After the oral uptake of *Ascaris* eggs, infective L3 stage larvae hatch from the eggs and undergo a hepato-tracheal migration through liver and lungs and finally back to the small intestine where they further develop to adult worms. During this hepato-tracheal migration, the larvae grow substantially and need to continuously adapt to the changing environment and cope with the host' immune responses. One could therefore expect a strictly controlled spatiotemporal gene expression pattern in these migratory larvae. The first insight into the developmental processes of the early *A. suum* stages was provided by Huang *et al*. [150]. A combination of suppressive-substractive hybridization and microarray analysis resulted in the identification of 498 cDNAs that were enriched in the infective L3s compared to the L3s present in the liver and lungs and the intestinal L4 stage, including genes that have known or predicted roles in embryonic and larval growth and development in *C. elegans*. However, information regarding the transcription patterns and biological processes taking place in the other larval stages during the hepato-tracheal migration are still largely missing.

Recently a 273 megabase draft genome of *A. suum* encoding for approximately 18,500 proteins was reported [151]. As part of this study, transcriptome datasets were produced from the L3-egg, L3-liver, L3-lung and intestinal L4 stage as well as from the somatic musculature and reproductive tract of adult male and female worms and subsequently used to assess the completeness of the genome assembly and gene predictions. The aim of this chapter was to further explore the transcriptome datasets of 4 different larval stages collected during the hepato-tracheal-intestinal migration (L3-egg, L3-liver, L3-lung and intestinal L4). The specific objectives were to identify both the highest transcribed and stage-specific transcripts for each larval stage, the metabolic changes and chemosensation pathways active in the larvae during their migration and, finally, the expression of potential molecular mimicry candidates by the larvae.

2.2 Materials and Methods

2.2.1 Ethics Statement

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine at Ghent University (Identification number EC2011/176) who have also approved the document.

2.2.2 Parasite material

A. suum eggs were obtained from the uteri of female worms, collected at a local slaughterhouse, and cultured in 0.1% $K_2Cr_2O_7$ for 28-30 days at 25°C. Once 90% of the eggs had become fully embryonated, they were used for infection experiments. The L3 larvae (L3-egg) were hatched from the eggs as previously described by Urban [152] and subsequently separated from eggshell fragments and other debris by baermannization. Three groups of 2 pigs were experimentally infected with infective *A. suum* eggs by oral intubation. The first group was inoculated with 500,000 eggs and euthanized 3 days p.i. to collect the liver stage larvae (L3-liver); the second group received 100,000 eggs and was euthanized 7 days p.i. to collect lung stage larvae (L3-lung); pigs of the third group received 30,000 eggs and were euthanized 14 days p.i. to collect intestinal stage larvae (L4). Larvae were separated from liver, lung tissue and small intestinal contents by baermannization. Nematodes were washed extensively in sterile physiological saline (37 °C), and subsequently, snap-frozen in liquid nitrogen and stored at -80°C until use.

2.2.3 RNA isolation, sequencing and assembly

The RNA isolation, sequencing and assembly were performed as part of a previous genome study [151]. Briefly, total RNA was obtained from *A. suum* L3-egg, L3-liver, L3-lung and L4 using the TriPure reagent (Roche) and the quality of the purified RNA verified by a 2100 BioAnalyser (Agilent). After processing the RNA samples according to the manufacturer's protocol (Illumina), RNA was subjected to paired-end RNA-seq using TruSeq chemistry on a HiSeq 2000

(Illumina) and assessed for quality and adaptor sequence. Transcripts were subsequently assembled from RNA-seq data using Oases [153].

2.2.4 Differential transcription analysis

All paired-end reads for each library constructed were aligned to the predicted *A. suum* gene set using TopHat, and quantitative levels of transcription (reads per kilobase per Million mapped reads (RPKM)) were calculated using Cufflinks [154]. Selection of the most abundant transcripts per stage (top 250) and stage specific (top 50) transcript genes was sorted according to the RPKM values. For the stage specific transcripts, only the genes with at least a 20-fold higher RPKM value compared with all other life stages were retained after filtering.

2.2.5 Quantitative real-time PCR

Quantitative real-time PCRs analyses were used to confirm the differential transcription indicated by the RNAseq data and were essentially performed as described previously [155]. Tubulin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were selected as housekeeping genes. The primer sets were designed by Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in Table 2.1.

Table 2.1: Sequence of gene-specific primers used for real-time PCR analysis

Gene ID	Homology	Forward and reverse primer 5' to 3'
GS_00826	Catalase	AACGACCACGTCTCCAAGAG GCACTTCTTCCTCAGGTTCG
GS_22077	Venom allergen 5	GTGGCTGGCTGAAGTTGTCT CCAACGTATCATCCGAACAG
GS_11597	Vacuolar amino acid transporter 4	CAGCCCACTGTATCCTCACA CAAACTCTCTGCGGTGAACA
GS_09830	Hyaluronidase-1	CGAATACCGACCCAAAACTG AACGATGGTCCCTTGCATAC
GS_08591	24 kDa protein of As22	GCTCACCGTATTCACCGAGT AGCGTAGTTGGCGTTCAATG
GS_14146	Sorbitol dehydrogenase	ACGATGGAAGATGGTGCTCT AGCCAGCTCCAAGTACGAGA
GS_13798	Cuticle collagen 12	GTTTGTGTGCCAATGCTCTG GTTTTGAGTGCGACACGAGA
GS_13054	Maltase-glucoamylase	CGGTCTATACGAGGCGAAAG TATCACCAAGCCAGTGTCCA

2.2.6 Functional annotation of coding genes

The Blast2GO program was used to obtain Gene Ontology (GO) annotations of the selected transcripts [156].The transcripts were mapped to respective pathways in *C. elegans* using KAAS [157] and their involvement in metabolic pathways mapped using the iPath tool [158]. In order to further characterize the *A. suum* transcripts, a series of BLAST homology searches were performed against different datasets, including NCBI, Wormbase, Nembase and Nematodes.net.

2.2.7 Molecular mimicry survey

A genome-wide survey with *A. suum* protein sequences was done as described previously [159]. In short, two *in silico* pipelines were used. One in which the predicted proteome of *A. suum* was broken down into overlapping fragments and subsequently screened for close hits in the human proteome. In parallel a similar procedure was carried out with the full-length protein sequences of *A. suum*. The proteomes of unrelated free-living eukaryotes were used to perform negative control analyses. In the current study, the reviewed Uniprot protein dataset of *Homo sapiens* was used as the 'host' dataset whereas the protein datasets of *Schizosaccharomyces pombe*, *Ciona intestinalis*, *C. elegans*, *Arabidopsis thaliana* and *Trichoplax adhaerens* were used as controls. Blast version 2.2.17 with BLOSUM 62 matrix was used to detect protein similarity.

2.3 Results and Discussion

2.3.1 A. suum larval transcriptome datasets

A total of 95,463,423 RNA-seq reads were produced for the different larval stages, 24,741,546 for the L3-egg stage, 23,763,236 for the L3-liver stage, 21,570,991 for the L3-lung stage and 25,387,650 for intestinal L4 stage (Table 2.2). The assembled sequence dataset is available on the Wormbase website (http://www.wormbase.org) under version WS230.

Table 2.2: Summary of the nucleotide sequence data for the larval stages of *A. suum*

Paired-end libraries	**L3-egg**	**L3-liver**	**L3-lung**	**L4**
Illumina (Solexa) reads				
Insert size (mean: stdev)	500bp	500bp	500bp	500bp
Read length	73	73	73	73
Total reads	24741546	23763236	21570991	25387650
Total data (Gb)	3.61	3.47	3.15	3.71
Expressed sequence tags (ESTs)				
Contigs	18511	18522	18533	18518

2.3.2 Top 250 most abundant transcripts in the different larval stages

Analysis of the abundant transcripts in the 4 different larval stages showed that the top 250 most highly transcribed contigs per stage accounted for approximately 60% of total transcription in each stage (63.44% for L3-egg; 61.43% for L3-liver; 65.55% for L3-lung, 57.17% for L4). Most of these contigs showed significant homology (e-value cut-off = 10^{-5}) to known proteins, 96.4% for L3-egg, 96.8% for L3-liver, 92% for L3-lung and 92.8% for L4. The distribution of the molecular function (level two) of the top 250 most abundant transcripts per stage according to the GO database is summarized in Figure 2.1 panel A. Distribution of GO terms indicated that binding (GO: 0005488), structural molecule activity (GO: 0005198) and catalytic activity (GO: 0003824) were the three major molecular function categories in each of the larval stages. Each of these top 3 functional categories contained at least 70 annotated genes, whereas genes involved in transporter activity, translation regulator activity, receptor activity, protein binding transcription factor activity, molecular transducer activity, enzyme regulator activity, electron carrier activity, channel regulator activity and antioxidant activity were less represented.

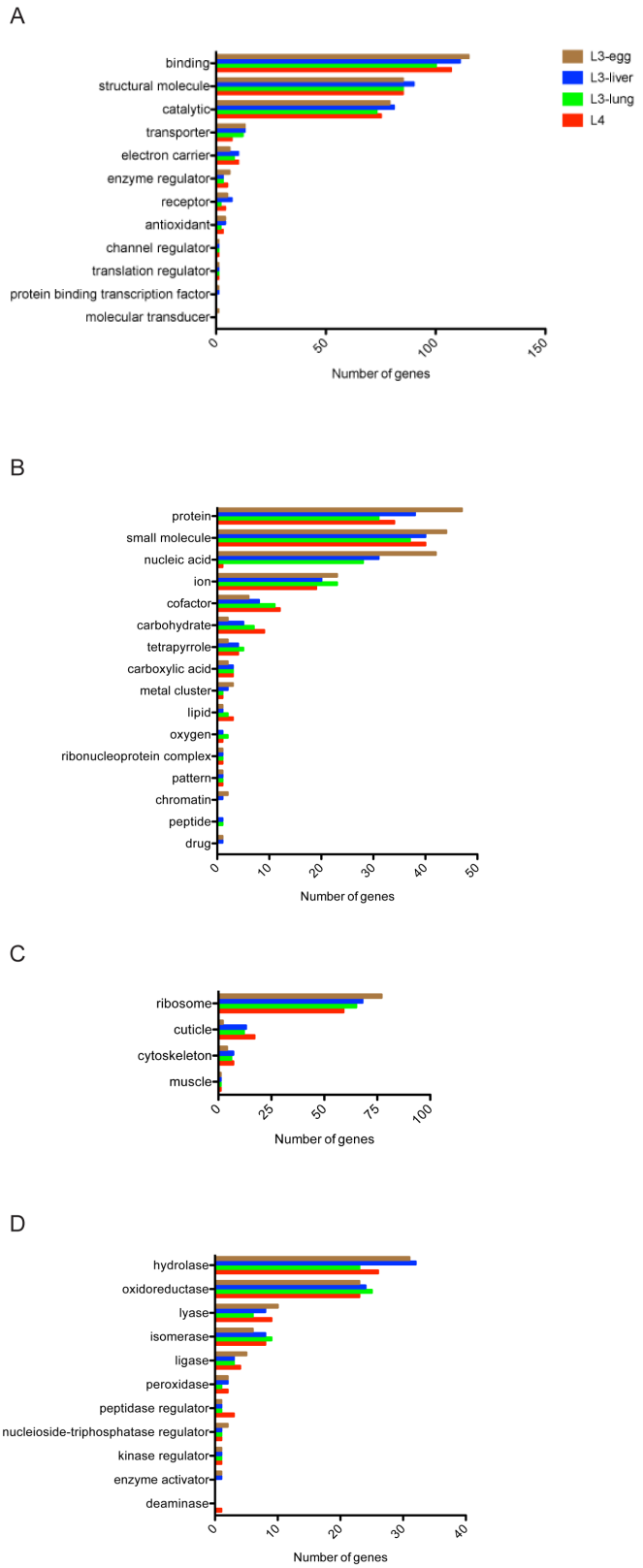

Figure 2.1: Molecular function distributions of the top 250 abundant larval transcripts. (A) Distribution of molecular function GO terms for the top 250 most abundant transcripts in the larval stages of *A. suum*. (B) Distribution of binding GO terms for the top 250 most abundant transcripts in the larval stages of *A. suum*. (C) Distribution of structural molecule activity GO terms for the top 250 most abundant transcripts in the larval stages of *A. suum*. (D) Distribution of catalytic activity GO terms for the top 250 most abundant transcripts in the larval stages of *A. suum*.

The genes associated with binding were subsequently further analyzed for their detailed functional category. The small molecule binding, protein binding, nucleic acid binding and ion binding were the predominant ones in each of the larval stages investigated (Figure 2.1 panel B). Within both the small molecule binding and protein binding terms, there were a significant number of heat shock protein-encoding genes, 7, 8, 5 and 6 in the L3-egg, L3-liver, L3-lung and L4 stage respectively. Heat shock proteins function as molecular chaperones and are involved in many cellular processes including protein trafficking, signal transduction, differentiation and development [160]. The expression of heat shock genes is developmentally regulated and can be induced by various stress factors, such as heavy metals, H_2O_2, heat and anaerobiosis.

For the genes associated with structural molecule activity, 4 functional categories at GO level three were examined (Figure 2.1 panel C). Among them, ribosome structural constituent was found to be the most abundant term in each larval stage. Ribosome structural proteins are functionally closely related and need to interact with each other physically to form a large protein complex known as the ribosome [161]. They are highly transcribed throughout the hepato-tracheal migration, likely reflecting the substantial rate of cell growth in these larval stages [162].

The second most abundant category of structural molecules was involved in the cuticle synthesis (Figure 2.1 panel C). Interestingly, there were 13, 12 and 17 collagens encoding genes in the top 250 most abundant transcripts of the L3-liver, L3-lung and the L4 stage, respectively, whereas only 2 were found in the L3-egg stage. The expression profile of the *A. suum* collagen genes present in the top 250 most abundant transcripts is represented in Table 2.3. In total, 24 transcripts showed significant homology (23-78%) to 17 different *C. elegans* collagens. The RPKM values suggest that these collagen genes were mainly transcribed from the L3-liver stage onwards. Interestingly, the transcription of some of them seems to be restricted to specific stages, such as contig GS_13304 in the L3-liver stage and GS_03348 and GS_06717 in the L4 stage. Although one

could assume that the high number of collagen encoding transcripts is involved in the fast growth of the larvae during their migration, research in *C. elegans* has shown that the production of collagens normally only starts from the mid-intermolt period onwards 4-2 hours prior to the actual molt [163]. In the case of *Ascaris*, the larvae molt to L4 around day 10 or 11 when they are back in the small intestine [164]. It therefore seems rather surprising to see such a high transcription of stage specific collagens approximately one week before their first molt in the intestinal tract, a timeframe that precedes the mid-intermolt period. The marked growth of the *A. suum* larvae between two molts may be achieved by simple stretching the cuticle, however previous work on *A. suum* has shown that there is also a marked increase in the thickness of both the basal and median layers of the cuticle when the larva develops into the adult stage [165-168]. Nevertheless, further studies are needed to explore whether active collagen production and thus cuticle synthesis is occurring in these larvae or whether the transcripts are being stockpiled for collagen production later on. It is for example possible that differences exist in the intermolt periods between *C. elegans* and *Ascaris*. The transcript levels of prolyl 4-hydroxylase, a key enzyme in collagen synthesis and indicative of actual collagen assembly, showed a peak in the L3 lung stages (results not shown). This would suggest that similar molting cycles exist in *Ascaris* but just slower compared to *C. elegans*. It is also interesting to note that the collagens transcribed in the larval stages of *Ascaris* are not the closest homologues of the collagens which have shown to be crucial for cuticle synthesis in *C. elegans* (i.e. not DPY-2, 3, 7, 8, 10, 13) [163].

Table 2.3: List of cuticle collagens identified in the top 250 most abundant transcripts

	RPKM					*C. elegans*	
Contig	**L3-egg**	**L3-liver**	**L3-lung**	**L4**	**length**	**gene**	**identity(%)**
GS_13304	1488.2	22411.6	7754.1	31.0	239AA	*col-154*	23
GS_15592	160.2	2556.6	1363.0	1114.8	288AA	*col-34*	57
GS_23251	245.9	4600.6	2487.8	1189.5	283AA	*col-184*	44
GS_04352	544.5	11483.7	8151.2	2284.5	263AA	*col-65*	55
GS_15987	169.2	4006.2	3122.1	1940.9	305AA	*col-14*	46
GS_16238	1534.1	41285.9	31329.1	1401.5	290AA	*col-73*	49
GS_12737	466.0	12333.3	11825.1	5333.0	367AA	*col-68*	62
GS_18402	635.2	17849.0	16707.4	10912.4	328AA	*col-184*	44
GS_00618	40.7	3263.4	4309.4	2325.0	243AA	*col-104*	35
GS_01804	79.7	3880.7	4985.6	1162.7	266AA	*col-109*	61
GS_08765	361.4	3939.8	5866.4	1503.6	311AA	*col-182*	37
GS_16792	92.3	5204.6	8397.4	1252.8	143AA	*col-48*	46
GS_22906	159.6	3864.7	4315.0	2098.0	160AA	*col-97*	53
GS_08970	708.2	12666.0	18270.7	13752.6	293AA	*col-184*	44
GS_09547	167.6	1790.5	1965.3	2401.4	88AA	*col-3*	78
GS_12116	18.2	1029.5	2492.6	3990.6	134AA	*col-150*	56
GS_18250	4.7	645.1	915.9	2013.2	207AA	*col-65*	31
GS_11610	63.7	738.5	253.0	1708.5	290AA	*col-73*	35
GS_14767	12.8	794.2	515.4	2772.3	326AA	*col-166*	72
GS_12783	28.4	1040.3	362.1	9335.6	216AA	*col-73*	64
L4_00638	1.8	395.4	230.8	2261.8	100AA	*col-184*	45
GS_21063	0.6	6.0	263.5	2081.7	334AA	*col-35*	64
GS_03349	0.1	0.4	0.8	989.4	322AA	*col-166*	72
GS_06717	12.0	0.4	5.4	1006.5	243AA	*col-77*	50

The highest RPKM value was underlined.

To gain further insights into the metabolic pathways active in the *A. suum* larvae during their migration, all the *A. suum* contigs of the top 250 that showed significant homology to *C. elegans* proteins (64.4% for L3-egg, 58.8% for L3-liver, 56.0% for L3-lung and 50.0% for L4) were analyzed by the KEGG pathways mapping. A total of 573 *A. suum* sequences, corresponding to 204 homologous genes in *C. elegans*, were assigned to 151 different KEGG metabolic pathways. The top 20 most highly represented pathways for each larval stage, categorized by the number of proteins mapped, are presented in Table 2.4. The metabolic pathways identified in each larval stage were also visualized using the ipath tool [158] (results no shown). Ribosome (n=73) was the main KEGG pathway that mapped to the sequences in each larval stage. In addition, a large number of

genes were observed to belong to the human disease pathways such as Alzheimer's disease (n=31), Parkinson's disease (n=29) and Huntington's disease (n=29). However, further analysis showed that these included proteins such as glyceraldehyde 3-phosphate dehydrogenase, NADH dehydrogenase, succinate dehydrogenase, cytochrome c oxidase, F-type H+-transporting ATPase subunit, cytochrome c, calmodulin and peptidyl-prolyl isomerase F (cyclophilin D), which are involved in a broad range of metabolic pathways active in these worms.

Table 2.4: Top 20 KAAS pathway analysis of abundant proteins of *A. suum* larvae

KEGG pathway	**Abundant proteins**				
	L3-egg	**L3-liver**	**L3-lung**	**L4**	**Total**
Ribosome	72	65	65	60	73
Oxidative phosphorylation	17	17	16	7	32
Alzheimer's disease	16	16	19	8	31
Parkinson's disease	15	16	17	7	29
Huntington's disease	15	16	17	7	29
Citrate cycle (TCA cycle)	5	10	9	11	12
Glycolysis / Gluconeogenesis	5	9	9	10	11
Valine, leucine and isoleucine degradation	2	5	10	7	10
Pyruvate metabolism	2	6	7	7	8
Cardiac muscle contraction	4	4	6	1	8
Glyoxylate and dicarboxylate metabolism	4	6	5	6	7
Propanoate metabolism	2	4	7	5	7
HIF-1 signaling pathway	5	5	6	5	7
Protein processing in endoplasmic reticulum	4	5	3	4	7
Carbon fixation pathways in prokaryotes	3	4	6	5	6
Phagosome	5	4	4	4	6
Fatty acid metabolism	0	2	5	2	5
Butanoate metabolism	0	3	5	3	5
RNA transport	5	2	1	1	5
Spliceosome	5	1	1	1	5

The analysis further indicated that different pathways involved in energy metabolism are activated in the larval stages, especially in the later larval stages. This could be a reflection of the increasing demand of energy of the larvae for their fast growth as well as for their migration through different host tissues. The pathways included the citrate cycle, glycolysis, valine, leucine and isoleucine degradation and pyruvate, glyoxylate and propanoate metabolism. Interestingly, enzymes involved in the metabolisation of starch and sucrose were highly represented in the top 250 most abundant transcripts of the L4 stage, whereas they appear to be less transcribed or even absent in the earlier larval stages.

2.3.3 Stage specific gene transcription

To identify the top 50 most abundant stage specific/enriched transcripts, the transcripts were sorted based on their RPKM values and subsequently filtered to retain transcripts with an arbitrary cut-off of at least a 20-fold higher RPKM value compared to all the other life stages. In order to validate the quantitative transcriptional difference of different contigs between the larval stages, as indicated by the RNAseq data, quantitative real-time PCR assays were performed for eight genes, i.e. a venom allergen (GS_00826) and a catalase (GS_22077) both enriched in the L3-egg stage, a vacuolar amino acid transporter (GS_11597) and a hyaluronidase (GS_09830) enriched in the L3-liver stage, a 24 kDa protein (GS_08591) and a sorbitol dehydrogenase (GS_14146) enriched in the L3-lung stage and, finally, a collagen (GS_13798) and a maltase-glucoamylase (GS_13054) enriched in the L4 stage. The results of the analyses, shown in Figure 2.2 panel B, basically confirmed the stage specific transcription patterns of the genes investigated.

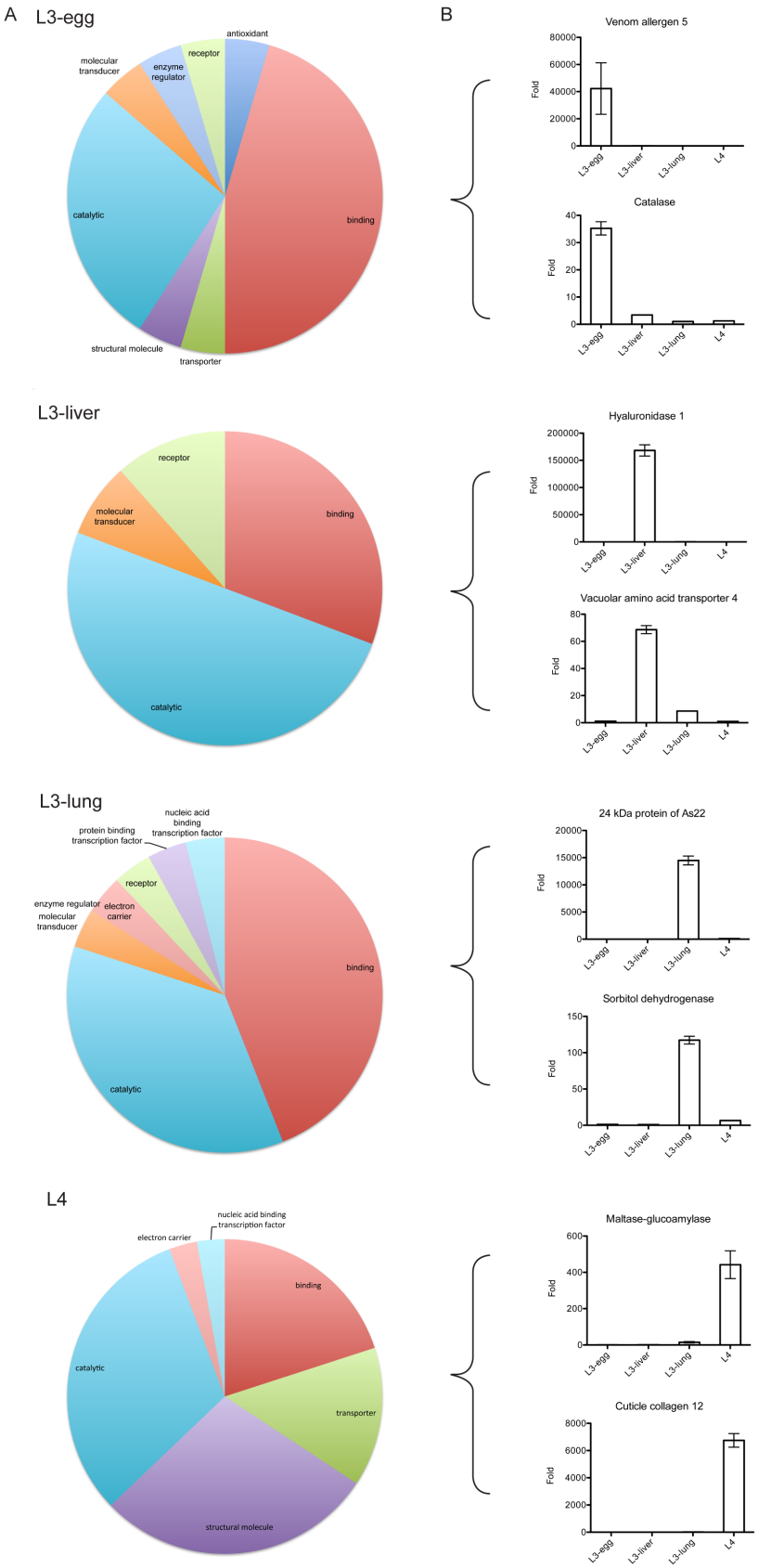

Figure 2.2: Molecular function distributions of the top 50 specific larval transcripts and qRT-PCR confirmation. (A) Distribution of molecular function GO terms (level 2) for the top 50 stage specific transcripts in the larval stages of *A. suum*. (B) Confirmation of the stage specific transcription pattern by qRT-PCR.

L3-egg stage specific transcripts:

Of the top 50 most abundant stage specific contigs of the L3-egg stage, 34% did not show any homology to other sequences present in the datasets. The genes that had GO annotations mainly belonged to the 'binding activity' (GO: 0005488) and 'catalytic activity' (GO: 0003824) categories (Figure 2.2 panel A). The 'binding activity' group was predominated by protein binding and ion binding functional genes, while the hydrolase genes leading the 'catalytic activity'.

Interestingly, amongst the L3-egg stage specific transcripts, there were two venom allergens belonging to the nematode SCP/TAPS protein family, i.e. GS_18986 and GS_00826 ranked 1st and 12th respectively. Although the exact function of SCP/TAPS proteins remains largely unknown, an important role in promoting nematode survival or development within the host, the angiogenic effects and the neutrophil chemoattractant activity has been suggested [169, 170]. Additionally, the potential of SCP/TAPS proteins as vaccine candidates has been demonstrated repeatedly in different species, including *A. caninum*, *Haemonchus contortus*, *Ostertagia ostertagi* and *N. americanus* [171-174]. Various studies have demonstrated that SCP/TAPS are amongst the most abundant proteins expressed and secreted during the transition from the free-living to the parasitic life stages [175-178], yet this seems to be not the case in *Ascaris*. In total, only 5 SCP/TAPS proteins are present in the top 250 most abundant transcripts of all the larval stages, and all of their RPKM values are below 3,000.

Also noteworthy is the catalase encoding transcript, ranked 38th in L3-egg stage specific transcripts. Catalases are haem-containing enzymes with a central role in the enzymatic detoxification pathway that prevents the formation of the highly reactive hydroxyl radical by decomposing H_2O_2 [179]. Catalases can function in two distinct modes: the rapid breakdown of hydrogen peroxide by catalytic activity or through the peroxidative activity by using H_2O_2 and other peroxides to oxidize a number of compounds [179]. Although the presence of catalase activity has been described in most parasitic nematodes investigated [180], the number of sequences identified that encode for catalases are so far

still limited [181, 182]. In *A. suum*, Lesoon *et al.* [183] previously described high catalase activity in unembryonated eggs and to a lesser extent also in homogenates prepared from different larval stages and adult tissues. The authors suggested that the high catalase activity in the eggs might protect the developing embryo from peroxide generated during the embryonation in an aerobic environment. Three catalase encoding transcripts were found in the *Ascaris* transcriptome dataset. However, further analysis showed that they are all encoding for the same catalase protein (P90682), which is identical to the sequence published by Eckelt *et al.* [181].

L3-liver stage specific transcripts:

Similar as with the L3-egg stage, 44% of the top 50 most abundant stage specific transcripts in the L3-liver stage did not show any homology to other known sequences. Analysis of the remaining sequences showed that most of them were associated with binding (GO: 0005488), catalytic activity (GO: 0003824), molecular transducer activity (GO: 0060089) and receptor activity (GO: 0004872). The 'catalytic activity' (n=14) category was the highest represented. Two sequences of interest that belonged to this category were chitinase (ranked 18th) and hyaluronidase (ranked 47th). Chitinases are enzymes that play a critical role in the hatching and exshethment process of nematodes by catalyzing the hydrolysis of beta-1, 4-*N*-acetyl-d-glucosamine linkages in chitin polymers [184-186]. In *A. suum*, chitinases were previously found in both the developing eggs and male sex organs [187, 188] and in the perivitelline fluid surrounding the infective larvae prior to hatching [184]. The transcription of a chitinase in the L3-liver stage might indicate that these enzymes are possibly also involved in other biological processes.

The expression of a hyaluronidase in the L3-liver stage is also noteworthy. Hyaluronidases belong to a family of enzymes that degrade hyaluronic acid. Hyaluronic acid is an anionic nonsulfated glycosaminoglycan that is a major component of the extracellular matrix [189]. In other parasitic nematodes (*A. caninum*, *Anisakis simplex*), hyaluronidases have been identified as potential host invasion factors utilized by the infective larvae for skin penetration or

gastrointestinal mucosal invasion [190, 191]. Based on these observations, Rhoads *et al.* [192] previously suggested that *A. suum* might utilize hyaluronidases to penetrate host tissue during the larval migration, which is in line with the data presented in this study.

L3-lung stage specific transcripts:

36% of the top 50 most abundant stage specific transcripts of the L3-lung stage showed no homology to any other known sequence. The GO analysis showed that binding (GO: 0005488) and catalytic activity (GO: 0003824) were again the two major molecular function categories in this larval stage. The 'binding activity' category was predominated by protein, ion, nucleic acid and small molecule binding proteins, whereas the genes encoding proteins with 'catalytic activity' were mainly hydrolase and oxidoreductase genes. The most striking observation in the list of the L3-lung enriched contigs is the high abundance of the contig ranked number 1, i.e. Liv_03570, with an RPKM value of 47,969, which makes this contig also the most abundant contig in the L3-lung RNAseq dataset. The contig sequence encodes a putative protein sequence of 79 amino acids long with no known homologues. Neither did it show any similarity/resemblance as any other contig in the *Ascaris* RNAseq database alone.

The appearance of globin (ranked 36th), belonging to the functional category 'binding', is also of interest. Globins are haem-containing proteins involved in binding and/or transporting oxygen. They belong to a very large and well-studied family that is widely distributed in many organisms, including nematodes [193]. In the *Ascaris* transcriptome dataset, 34 putative globin transcripts can be found. Most of the *Ascaris* globins (24 out of 34) showed high homology to the 33 described globins in *C. elegans* (results not shown). *Caenorhabditis* globins can be separated into two well-resolved classes, based on their sequence similarity [194]. *C. elegans* GLB-1, as well as its 4 orthologs in *A. suum* (myoglobin, haemoglobin, globin-like protein and globin-type protein9), belongs to the first class, whereas all remaining *C. elegans* globins and novel orthologs thereof identified in parasitic nematodes are class 2 globins. In *C. elegans*, Class 2 globins show huge variability of intron positions and generally

have disproportionally short branch lengths whereas this is not the case in Clade 1 globins. This suggests a faster rate of evolution in Clade 2 globins. It is therefore likely that these globins could have acquired novel and highly specific functions [195]. In the case of the *A. suum* lung stage larvae, the production of this high oxygen affinity globin could potentially be involved in the prevention of the toxic effects of oxygen in the oxygen rich environment of the lung [196].

The thioredoxin, ranked 10th and categorized under 'catalytic activity', could potentially also be involved in detoxification processes. Thioredoxin belongs to a large family of anti-oxidant proteins, including glutathione peroxidases, peroxiredoxins and translationally controlled tumor protein-like proteins, that are produced by a wide range of organisms in the defence against toxic hydroxyl radicals that can damage proteins, lipids and DNA [197]. In parasitic nematodes, these molecules are potentially produced to cope with reactive oxygen species (ROS), generated either during their own cellular metabolism and/or by the host immune response [198].

L4 stage specific transcripts:

In contrast to the different L3 larval stages, 94% of the L4 stage specific transcripts showed homology to sequences present in public databases. Besides binding (GO: 0005488) and catalytic activity (GO: 0003824), which were also major molecular function categories in the L3 larvae, structural molecule activity (GO: 0005198) (n=10) and transporter activity (GO: 0005215) (n=5) were another two predominant categories represented in the top 50 most abundant stage specific transcripts of the L4 stage.

Among the transcripts categorized under the term 'binding' were 3 C-type lectins (ranked 4th, 12th, and 47th). C-type lectins are carbohydrate-binding proteins that are widely distributed throughout the animal kingdom. In parasites, C-type lectins have been suggested to play a role in immune evasion [199]. The recent report of similarity between host dendritic cell receptors and C-type lectins from *A. suum* suggests that *Ascaris* may utilize lectins to bind to carbohydrate moieties on the host cells to avoid pathogen recognition mechanisms in hosts [200]. By

masking the L4 larvae, C-type lectin permits the worm to establish under the host's immune system in gut, where they live in their final development stage.

The 10 genes that were annotated to the 'structural molecule activity' term all showed homology to nematode cuticle collagens. The stage enriched transcription pattern of some collagens is consistent with our previous observation (Table 2.3). For the 5 transcripts associated with transporter activity, the homologue of aquaporin is of particular interest. Aquaporins are intrinsic membrane proteins that selectively transport water, glycerol and some small solutes across cell membranes [201]. Studies showed that *A. suum* transports water directly across the cuticle to withstand great fluctuations in osmotic pressure [202]. It is possible that the aquaporin identified here plays an essential role in this process.

In the group of transcripts without GO annotation, it is also interesting to note that two antibacterial proteins (cecropin and ASABF) are specifically transcribed in the L4 stage larvae. The antibacterial activity of cecropin was reported against Gram-negative bacteria, whereas the specificity of the ASABF (*Ascaris suum* antibacterial factor) peptide was mainly directed against Gram-positive organisms [203-205]. Therefore, the combination of these two peptides could provide L4 larvae with a broad-spectrum protection against the intestinal flora. Furthermore, it is also interesting from the evolutionary and ecological point of view that the host could potentially take advantage of these nematode peptides for protection against potentially harmful bacteria [205].

2.3.4 Metabolic pathways

For a more complete analysis of the metabolic changes during larval development, the whole *Ascaris* dataset was searched against the *C. elegans* KEGG metabolic pathways, including general energy metabolism, lipid/fatty acid metabolism and carbohydrate metabolism. The analysis resulted in the identification of 203, 171 and 257 *Ascaris* homologues potentially involved in these three metabolic pathways, respectively (results not shown). Analysis of the transcript levels of genes involved in general energy metabolism indicated that

they accounted for approximately 7% of the total transcriptome in the L3-egg stage larvae. This proportion further increased to 8% in the L3-liver and L3-lung stages and approximately 9.5% in the L4 larvae. The same calculation was also performed for the genes involved in the lipid/fatty acid and carbohydrate metabolic pathways separately. The results indicate that the genes involved in carbohydrate metabolism are much higher transcribed, accounting for almost 10% of the total transcriptome of the L4 stage larvae, compared to the genes involved in lipid/fatty acid metabolism (Figure 2.3).

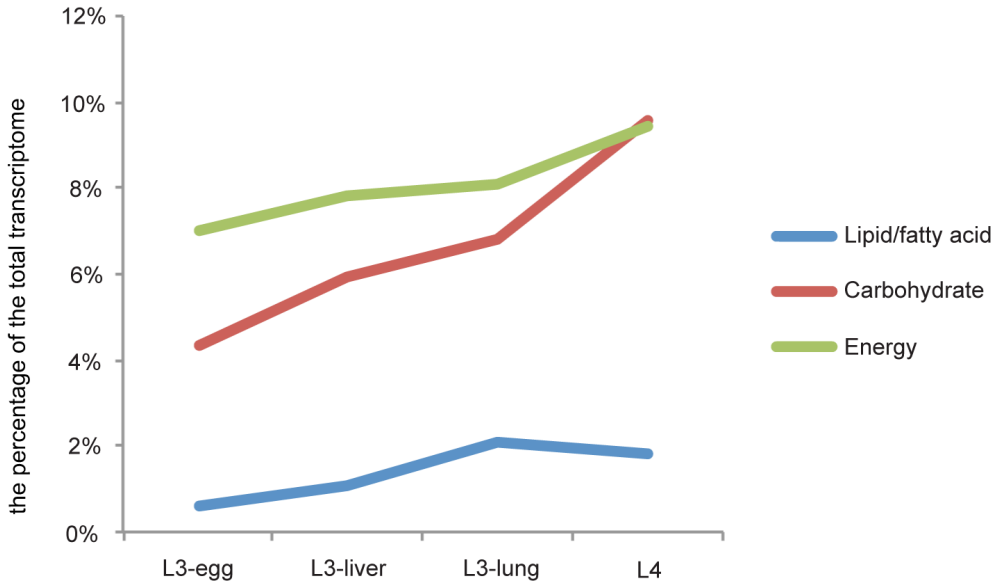

Figure 2.3: The ratio of the total RPKM values of all the genes involved in energy metabolism, lipid/fatty acid metabolism and carbohydrate metabolism compared to the total transcription.

These observations are in line with the analyses of the most abundant transcripts described above. All these results suggest that the degradation of complex carbohydrates forms an essential part of the energy metabolism of this parasite, especially once it establishes in the small intestine. Whether similar processes are active in other worm species with a similar lifestyle is still unclear and warrants further research.

2.3.5 Chemosensory pathways

Since molecules associated with chemosensory pathways, in particular olfactory, are potentially important in the migration of *A. suum* larvae into and through

different tissues and organs, one of the objectives of this study was to search the *A. suum* transcriptome database for genes potentially involved in these pathways. Therefore, the *A. suum* dataset was BLAST searched with 40 different *C. elegans* genes involved in chemosensation [206]. Homologues were identified for 29 proteins, although some of them appeared to be not very conserved (Table 2.5).

In terms of the olfactory neuron proteins, numerous homologues could be identified in *A. suum* (underlined in Table 2.5). These molecules were proposed to function as receptors, signal transducers and regulators in three known *C. elegans* olfactory neurons, i.e. AWA, AWB and AWC [206]. The RPKM values of these olfactory neuron molecules show that their transcription is relatively high in the early larval stages and then gradually decrease as the larvae reach the lungs. This observation is in line with a previous hypothesis that olfactory chemosensation was utilized by the larvae to navigate during the hepato-tracheal migration [151]. In the L4 larvae however, many of these genes seem to be transcriptionally activated again, suggesting that they might also play a role in the migration of the larvae within the small intestine. Among the olfactory neuron molecules, the highest transcribed gene was a *ttx-4* homologue, which, in addition, also seems to have undergone a gene duplication event in *A. suum*. In *C. elegans*, the *ttx-4* gene encodes a protein kinase C of the epsilon/eta class [207], and is required for the regulation of signal transduction in various sensory neurons for temperature, odor, taste, and high osmolarity.

Apart from olfactory proteins, *daf-21* also seems to be important in *A. suum*, with high transcript levels observed in all the larval stages. *daf-21* encodes the heat-shock protein 90, a chaperone with numerous specific protein targets. Previous study showed that the heat-shock protein 90 regulates the levels of cGMP, which is the prominent second messenger in *C. elegans* chemosensory transduction [208]. Finally, analysis of the RPKM values showed that *gpc-1* is highly upregulated in the lung stage. In *C. elegans*, *gpc-1* encodes a heterotrimeric guanine nucleotide-binding protein gamma subunit, expressed specifically in sensory neurons that block salt adaptation [209]. The precise reason for the

enrichment of *gpc-1* in the lung stage larvae remains unclear.

Table 2.5: List of *C. elegans* chemosensory homologues in *A. suum* dataset

Chemosensory molecules				**RPKM value**			
C. elegans	***A. suum***	**e value**	**Homologue**	**L3-egg**	**L3-liver**	**L3-lung**	**L4**
adp-1	None						
daf-11	GS_12046	e-172	65%	2.13	0.05	0.03	0.02
daf-21	GS_17449	0	86%	3432.23	2446.33	944.89	2952.25
egl-4	GS_07162	0	88%	25.99	38.76	14.01	22.42
fat-3	None						
gcy-35	None						
gcy-36	None						
goa-1	GS_21442	0	98%	24.29	11.00	0.07	6.51
gpa-1	GS_15380	e-102	77%	6.77	14.23	0.08	2.10
gpa-2	None						
gpa-3	GS_01671	0	96%	0.14	0.15	3.17	0.25
gpa-4	GS_15208	e-140	77%	17.23	11.51	0.54	4.86
gpa-5	None						
gpa-6	GS_04115	0	75%	0.05	0.05	0.08	0.05
gpa-8	None						
gpa-9	None						
gpa-10	None						
gpa-11	GS_05059	e-111	71%	3.24	0.05	0.07	0.04
gpa-13	GS_13737	0	58%	43.58	12.28	0.06	0.63
gpa-14	None						
gpa-15	GS_15970	0	72%	0.06	0.06	0.09	0.05
gpc-1	GS_18764	0	80%	113.56	17.86	595.57	1.13
grk-2	GS_23403	0	93%	11.46	11.69	25.27	20.52
kin-29	GS_16066	e-122	70%	16.21	12.10	2.86	9.82
let-60	GS_12465	0	88%	4.10	16.23	7.87	17.47
npr-1	GS_10409	0	63%	0.22	0.04	0.06	10.86
ocr-2	GS_18744	0	68%	37.87	13.84	0.28	1.46
odr-1	GS_12010	e-158	62%	0.02	0.02	3.57	0.30
odr-3	GS_15633	e-133	91%	1.60	1.12	0.08	1.71
odr-4	GS_10493	0	57%	5.21	19.18	0.84	4.63
odr-10	GS_07734	0	51%	1.34	0.06	0.09	0.06
osm-9	GS_22862	0	75%	1.00	0.02	0.51	0.09
osm-10	None						
qui-1	GS_17991	0	63%	0.02	1.26	0.02	0.01
sdf-13	GS_22659	e-104	91%	2.02	5.19	2.96	12.25
str-2	None						
tax-2	GS_03142	0	77%	1.75	0.45	0.04	5.25
tax-4	GS_13536	e-174	66%	0.93	1.90	0.04	0.03
tax-6	GS_10211	0	97%	6.22	2.55	0.17	6.38
ttx-4	GS_09285	0	91%	2.67	14.59	0.06	5.77
ttx-4	GS_20022	e-156	84%	388.81	328.41	114.39	463.46

These homologues related to olfactory chemosensation were underlined.

2.3.6 Molecular mimicry

In an attempt to identify potential immunomodulators, we screened the complete *Ascaris* transcriptome dataset for molecular mimicry candidates. These were defined as parasite proteins with (i) a blastp score above 100 to the best hit

in the human proteome and (ii) a score in *H. sapiens* of at least two-fold higher than the best score achieved in negative control proteomes. Using whole protein sequences, 12 molecular mimicry candidates were identified in the *Ascaris* dataset. Of particular interest is GS_13355, which shows similarity to a host protein that belongs to the suppressor of cytokine signaling family (SOCS) (Figure 2.4). SOCS proteins were known as crucial physiological regulators of both innate and adaptive immunity [210]. These molecules positively or negatively regulate macrophage and dendritic-cell activation and are essential for T-cell development and differentiation [210]. SOCS proteins were also shown to be involved in the immune response to intracellular parasites (*Plasmodium berghei* [211] and *Toxoplasma gondii* [212]). Zimmermann *et. al.* [212] suggested that the induction of SOCS proteins within phagocytes, due to infection with *T. gondii,* contributes to the parasite's immune evasion strategies. A previous study on the genome-wide identification of molecular mimicry candidate in *Brugia malayi* also resulted in the identification of a SOCS protein [159]. GS_11563, another interesting mimicry molecule, shows similarity to a plasma glutamate carboxypeptidase. An orthologue (Leucyl aminopeptidase, ES-62) in *Acanthocheilonema viteae* (Uniprot ID O76552, 55.0% identity) seems to modulate key signal transduction pathways associated with immune cell activation and polarization [213]. Finally, it is also interesting to note that the approach followed in this study resulted in the identification of totally different proteins compared to the approach followed in a previous study [151] in which in a first step putatively secreted proteins were identified followed by a BLAST search. In order to have an in-depth analysis of ES proteins, an *in vitro* study will be conducted in the next chapter.

```
SOCS5       WKVHTQIDYIHCLVPDLLQITGNPCYWGVMDRYEAEALLEGKPEGTFLLRDSAQEDYLFS   415
GS_13355    YVVHTSVDYTNCLVPAQDRITASSYYWGVMDRYEAEALLDGKPEGTFLLRDSAQSEYLFS   228
            + VHT +DY +CLVP   +IT +  YWGVMDRYEAEALL+GKPEGTFLLRDSAQ +YLFS

SOCS5       VSFRRYNRSLHARIEQWNHNFSFDAHDPCVFHSSTVTGLLEHYKDPSSCMFFEPLLTISL   476
GS_13355    VSFRRYKRTLHARIEQKNHRFSFDFSDTSIFSAETITDLIAYYKDPAKCLFFEPQLSIPL   288
            VSFRRY R+LHARIEQ NH FSFD  D  +F + T+T L+ +YKDP+ C+FFEP L+I L

SOCS5       NRTFPFSLQYICRAVICRCTTYDGIDGLPLPSMLQDFLKEYHYKQKVRV   525
GS_13355    PRNFVFSLQHICRARIASLTTYDGVNELSLPVALKQYIQEYFYKHPVKT   337
            R F FSLQ+ICRA I   TTYDG++ L LP  L+ +++EY YK  V+
```

Figure 2.4: ClustalW aligment of GS_13355 to SOCS5 from *Sus domesticus*. The SH2 domain is shaded in yellow, the SOC box domain in green. The N-terminal parts of the two proteins did not share any similarity and are therefore not shown in the alignment.

CHAPTER THREE

Proteomic analysis of the excretory-secretory products from larval stages of *Ascaris suum*

Base on:

Wang T., Van Steendam K., Dhaenens M., Vlaminck J., Deforce D., Jex A., Gasser R.B., Geldhof P. Proteomic analysis of the excretory-secretory products from larval stages of *Ascaris suum* reveals high abundance of glycosyl hydrolases. PLOS Neglected Tropical Disease 7(10): e2467.

3.1 Introduction

During the initial stage of infection, *Ascaris* worms undergo molts and larval migration in several well-defined organ systems, i.e. liver, lungs and small intestine. The proteins produced and presented at the parasite-host interface during these different phases of tissue invasion and migration are inferred to play a critical role in the induction and development of immune responses [214]. Such proteins can be present on the outermost layers of the cuticle and in the excretory-secretory (ES) products, which are mainly released from the cuticular surface, specialized excretory/secretory organs and the worm intestine [214, 215]. To date, little is known about these components from *A. suum*. Limited by technical and practical constraints, earlier studies of ES products from *A. suum* were mainly focused on exploring their chemical composition, ultrastructure and immunological role [216-220]. Recently, with major developments in mass spectrometry and genomic technologies, many of the previous challenges and limitations in the proteomic analysis of parasite ES proteins have been overcome, and have led to the characterisation of ES proteomes for parasitic nematodes including *A. caninum*, *B. malayi, H. contortus*, *Teladorsagia circumcincta* and *Trichinella spiralis* [176, 221-227]. Nonetheless, there has been no profound proteomic analysis of *Ascaris* ES products at critical stages of development.

The aim of this chapter was to characterize the ES proteins of three different larval stages of *A. suum* (i.e. L3-egg, L3-lung and L4) using tandem mass-spectrometry combined with the recently completed *A. suum* genome for annotation [151]. In addition, transcriptomic datasets of the larval stages [151] were used to investigate transcription of genes encoding some of the proteins identified in the ES products from the three larval stages.

3.2 Materials and Methods

3.2.1 Ethics Statement

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical

approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine at Ghent University (Identification number EC2011/176) who have also approved the document.

3.2.2 Parasite material

Adult worms of *A. suum* were collected from naturally infected pigs at the local slaughterhouse as part of the normal work at the abattoir. Subsequently, male and female worms were dissected and the intestine, reproductive system and cuticle collected and stored at -80°C until use. Eggs of *A. suum* were obtained from the uteri of female worms, and cultured in 0.1% $K_2Cr_2O_7$ for 28-30 days at 25°C. After 90% of the eggs had become fully embryonated, the infective L3s (L3-egg) were hatched from the eggs as described previously by Urban and Douvres [152] and then separated from eggshell fragments and other debris by baermannization.

Two groups of two pigs were experimentally infected with larvated eggs of *A. suum* by gavage. Pigs of group one were each inoculated with 500,000 eggs and euthanized seven days p.i. in order to collect the lung stage larvae (L3-lung), whereas pigs of group two each received 30,000 eggs and were euthanized 14 days p.i. to collect intestinal stage larvae (L4). L3-lung and L4 were separated from lung tissue and small intestinal contents of host by baermannization, respectively.

3.2.3 Preparation and analysis of ES products

All three larval stages (L3-egg, L3-lung and L4) were cultured for five days in RPMI 1640 medium with 10 mM L-Glutamine (GIBCO, Invitrogen) containing 0.2 mg/ml gentamycin (10 mg/ml GIBCO, Invitrogen), 1% amphotericin B (250 µg/ml, Sigma), 1 mg/ml streptomycin (Sigma) and 1,000 U/ml penicillin (Kela pharma). The viability of larvae was checked daily and the culture fluid was collected every 24 h and filtered through a 0.22 µm filter (PALL Corporation). After 5 days, the filtrates were pooled and then concentrated and dialysed against phosphate-buffered saline (PBS) at 4°C using filters (Amicon, YM-10 membranes, Millipore). Proteins were precipitated through the addition of 6

volumes of cold acetone for 18 h at -20°C. The proteins were pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was resuspended in PBS and stored in aliquots at -80°C. For SDS-PAGE analysis, protein samples (20 μg per lane) were mixed with loading buffer (2% SDS, 50 mM Tris HCl and 5% β-mercaptoethanol), boiled for 5 min and then separated on 12% SDS-PAGE gels using a standard procedure [228]. After staining with Coomassie Brilliant Blue (Invitrogen), the entire gel lane was sliced in 10 equal pieces (horizontally) and used for subsequent liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis.

3.2.4 In-gel and in-solution tryptic digestion and LC-MS/MS analysis

Tryptic in-gel digestion was performed as described previously [229]. In brief, to ensure better transfer of buffers, each protein band was cut into 1 mm^2 portions, washed twice in 50% acetonitrile with 25 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol in 25 mM ammonium bicarbonate, alkylated with 100 mM iodoacetamide in 25 mM ammonium bicarbonate and digested with trypsin (200ng per band) at 37°C for 18 h. Peptides were extracted with acetonitrile and dried in a Speedvac.

The in-solution digestion was performed as previously described [230]. In brief, 10 μg of the acetone-precipitated ES proteins were resuspended in 20 μl of 0.5 M triethylammonium bicarbonate buffer, reduced with 2 μl of 10 mM dithiothreitol and incubated at 60°C for 1 h. Subsequently, 1 μl of 200 mM methyl methanethiosulfonate in isopropanol was added and incubated for 10min at room temperature. The solution was digested with trypsin (resuspended in triethylammonium bicarbonate) in at a ratio of 1/50 (amount trypsin/protein) overnight at 37°C.

Dried peptides were dissolved in 40 μl 0.1% formic acid (FA) and 20 μl was desalted for 10 min on a C-18 pre-column (C18 PepMap100, 5 μm x 5 mm, i.d. 300 μm Dionex) with 0.1% FA. Separation was performed by means of reversed phase nano-HPLC (25 cm PepMap C18 analytical column, Dionex) at 60°C using a linear gradient of H_2O: ACN (97:3, 0.1% FA) to H_2O: ACN (20:80, 0.1% FA) at 300

nl/min over 70 min. The different peptides were analyzed on an ESI Q-TOF Premier (Waters, Wilmslow, UK) in a data dependent mode, with automatic switching between MS and MS/MS for up to 7 higher charge ions, when the intensity of the individual ions rose above 50 counts per sec. Fragmentation of the precursors was performed by means of CID. The capillary voltage was set at 1.9 kV, and the cone voltage was set at 100. M/z ratios for MS ranged between x and y and for MS/MS between x and y. M/z ratios selected for MS/MS were excluded for 150 sec. A custom collision energy profile was used.

3.2.5 Database searching and sequence analysis

Data were searched against an in-house *Ascaris* sequence database (18,542 protein entries), which is based on the recently published *A. suum* genome [151], using the search engine Mascot Daemon (v.2.3, Matrix Science, London, UK), allowing a maximum of one miscleavage. Carbamidomethyl (C) was specified as fixed modification and carbamidomethyl (N-term), deamidated (NQ) and oxidation (M) were considered as variable modifications for in-gel digest. For in solution digests, methylthio (C) was selected as the fixed modification, and deamidated (NQ) and oxidation (M) as variable modifications. An error-tolerant Mascot search was performed as well. The peptide tolerance and MS/MS tolerance were set to 0.35 Da and 0.45 Da, respectively. Only the most parsimonious group of protein identifications were reported from the identified proteins, and the identification threshold was set at $p < 0.01$. For the proteins that were annotated based on only one peptide, the identification threshold was set at $p < 0.0001$. An estimate of the relative abundance of the predicted proteins in the trypsin digestion was assessed using the Exponentially Modified Protein Abundance Index (emPAI) [231] together with the MS score, sequence coverage, detected peptides numbers. For redundant identifications, the emPAI value from the hit with the highest score was considered. The Gene Ontology (GO) database was used for inferring the molecular function of individual proteins identified. The protein sequences were analysed for the presence of signal peptides and transmembrane regions with SignalP 3.0 and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. The subcellular localization was predicted with SecretomeP 2.0. The sequences of the identified

proteins were then used to BLAST search the *A. suum* genome to identify homologous sequences. This was done through the WormBase (http://www.wormbase.org/) (E-value threshold=1E-16).

Amino acid sequences of selected eukaryotic glycosyl hydrolases listed in the CAZy database (http://www.cazy.org/) were downloaded and used for multiple alignment and consecutive phylogenetic analyses. These sequences included: *Homo sapiens* alpha acid glycosidase (AAG) (P10253), dual catalytic sucrase-isomaltase (SUIS) (P14410), maltase-glucoamylase (MGA) (O43451), alpha glucosidase AB (GANAB) (Q14697), alpha glucosidase C (GANC) (Q8TET4); *Bos taurus* AAG (Q9MYM4); *Mus musculus* AAG (P70699); *Coturnix japonica* AAG (O73626); *Oryctolagus cuniculus* SUIS (P07768); *Suncus murinus* SUIS (O62653); *Rattus norvegicus* SUIS (P23739); *Sus scrofa* GANAB (P79403); *Drosophila melanogaster* AAG-like (Q7KMM4) and *Caenorhabditis elegans* AAGR1-4. The protein sequences were subjected to MUSCLE alignment (http://www.ebi.ac.uk/Tools/msa/muscle/), and alignments verified and visually checked and edited, as required, in Jalview (http://www.jalview.org/). The program ClustalX 2.0.10 was used to generate phylogenetic tree following analysis using the neighbour-joining method (1,000 replicates) [232]. Finally, the program WebLogo application (http://weblogo.threeplusone.com/create.cgi) was used to provide a graphical representation of the amino acid homology around the catalytic sites of some of the glycosyl hydrolases of *A. suum* and *C. elegans*.

3.2.6 RNA extraction and quantitative real-time PCR (qPCR)

Total RNAs from larvae and adult worm tissue samples were isolated using TRIzol (Invitrogen), followed by further purification with the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. An on-column DNase digestion was performed using the RNase-free DNase set (Qiagen) to remove any possible genomic DNA. The RNA concentrations were determined (NanoDrop ND-1000 spectrophotometer, NanoDrop Technologies) and its quality was verified (Experion™ Automated Electrophoresis System, Bio-Rad). For all samples, the RNA quality indicator (RQI) calculated (Experion™ software, Bio-

Rad) was 8.0, demonstrating high RNA integrity. The qPCR analyses were performed as described previously [155]. Tubulin and GAPDH were selected as housekeeping genes. The primer sets used were designed by Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in Table 3.1.

Table 3.1: Nucleotide sequences of the primers used in the qRT-PCR assays

Gene ID	Homology	Forward primer 5' to 3'	Reverse primer 5' to 3'
GS_10488	Serpin-like protein	GTGCCGATGGGAAAACTAAG	GCATCCTTTAGCGAGAGACG
GS_18934	Maltase-glucoamylase	CTTCTTCGAGTTTCCGAACG	GAGAGGAGCGTTGAGGAATG
GS_19777	Sucrase-isomaltase	ACGAGAGAAAGCGGATACCA	GAACTGAAGCCAGCGTTTTC
GS_20130	GAPDH	CGGTTGTATCGACGGACTTT	TGCTGATGTAAGCGATGAGG
GS_23993	Tubulin	CGAGAGGGTTGAAGATGAGC	ATGTTGCTCTCCGCTTCTGT

3.2.7 Analysis of differential transcription

A transcriptome dataset was generated from the L3-egg, the L3-liver, the L3-lung and the intestinal L4 stages as part of a previous study [151]. Briefly, following RNA-seq, all paired-end reads for each library constructed were aligned to the predicted *A. suum* gene set using TopHat. Levels of transcription (RPKM) were calculated using Cufflinks [154]. To obtain the RPKM values for genes of interest, accession numbers from the *A. suum* genome were used to search the transcriptomic datasets.

3.2.8 Protein extraction and enzymatic assays

Protein extracts of larval stages or adult worm tissues were produced by grinding the frozen material to a fine powder in a liquid nitrogen-cooled pestle and mortar. The powder was sequentially subjected to a two-step process with reagents of increasing solubilising power [233]. For the water-soluble protein fraction, 4 ml of PBS, pH 7.4, were used to resuspend the powder for 2 h at 4°C by gentle 'head-over-head' mixing. The insoluble material was pelleted by centrifugation at 120,000 *×g* for 15 min and the supernatant retained. For the water-insoluble protein fraction, the pellet was incubated at 22°C for 3 h using an extraction buffer consisting of 5 M urea (Sigma), 2 M thiourea (Sigma), 2%

CHAPS (Sigma) and 2% SB3-10 (Sigma) in 40 mM Tris, pH 7.4. The supernatant was collected, as described for the water-soluble protein fraction. A general use cocktail of protease inhibitor (Sigma) was added to each extracts to avoid proteolytic degradation. Protein concentrations were measured with the Bradford reagent (Sigma), and proteins stored at -80°C.

The glycosidase assays were conducted by incubating 5 μg of protein extract with 30 mM of substrate at pH 6.5 for 40 min at 37°C. Reactions were quenched by the addition of 20 μl of 3 M Tris. The glucose was quantified using the Glucose Assay Kit (Sigma). The substrates used in the assays included maltose, lactose and sucrose. Each analysis was performed three times, and the results presented as the average of the three readings. For statistical analysis, the unpaired student t-test was used to test differences in activity between the different protein homogenates. The level of significance for analyses was set at $P \leq 0.05$.

3.3 Results

3.3.1 Proteins profiles of the excretory/secretory material

The protein profiles of the ES products from each of the three larval stages of A. suum, displayed by SDS-PAGE and Coomassie staining, are shown in Figure 1. The analysis revealed a complex and distinct banding pattern for the ES of three individual stages. Most ES proteins from L3-egg were distributed between 10-120 kDa, whereas those of L3-lung were mainly between 30 and 100 kDa, with a smear above 40 kDa. L4 ES represented a complicated profile, with major bands between 37 and 150 kDa, and some fainter bands in the 20-30 kDa range.

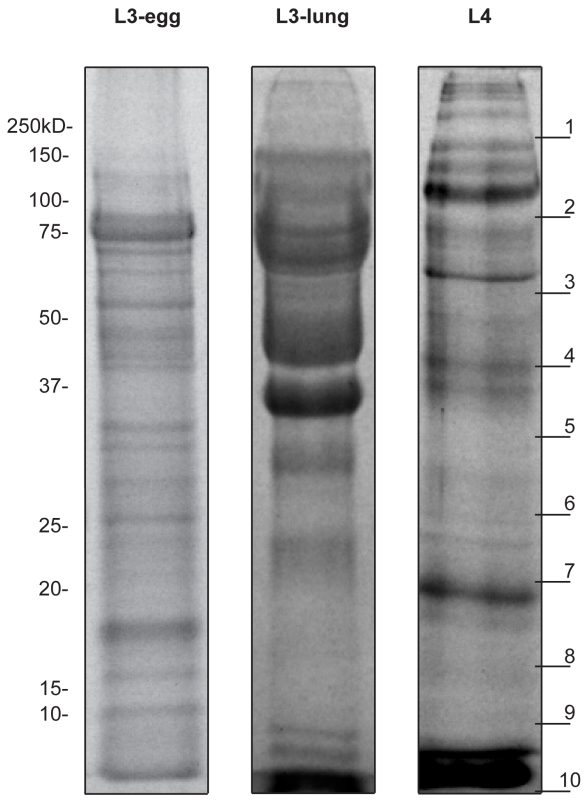

Figure 3.1: Protein profile of the *A. suum* L3-egg, L3-lung and L4 ES products displayed on a 12% SDS-PAGE stained with Coomassie blue. Each lane was loaded with 15 µg of protein. Molecular weight markers are indicated to the left. The 10 gel slices used in the trypsin digests are indicated on the right.

3.3.2 Protein identifications

Mascot searches of the MS/MS spectra for both the in-gel and in-solution approaches yielded 20, 45 and 58 protein identities within ES products of L3-egg, L3-lung and L4 stages, respectively. The full lists of proteins identified are provided in Tables 3.2 (L3-egg), 3.3 (L3-lung) and 3.4 (L4). Most ES proteins detected were inferred to be stage-specific [85% (n=17) for L3-egg, 69% (n=31) for L3-lung and 74% (n=43) for L4], and 15 proteins identified in ES products were shared by at least two larval stages. The identities of proteins shared by all three stages are given in Figure 2. ES products from L3-lung and L4 shared 14 proteins, representing 31% and 24% of their sub-total, respectively, whereas the L3-egg shared only 2 and 3 proteins with L3-lung and L4, respectively. Finally, two proteins shared by all three ES samples included a 14-3-3-like protein and a serpin (Figure 3.2).

There are two types of protein secretion, i.e. classical secretion and non-classical

secretion. The classical protein secretion is *via* secretory portals at the cell plasma membrane [234], whereas the non-classical protein secretion through various non-classical pathways [235], such as direct translocation of proteins across the plasma membrane, blebbing, lysosomal secretion and release *via* exosomes derived from multivesicular bodies. *In silico* prediction of classical secretion and non-classical secretion showed that 9 (45%), 25 (56%) and 42 (72%) of the identified proteins from L3-egg, L3-lung and L4 ES products, respectively, were predicted to be either a classical or non-classical secreted protein (Tables 3.2-3.4).

All proteins identified were subsequently categorized based on their molecular function, according to information from the GO database. Assigned were: metabolic pathway, structural, motor activity, binding, other functions and proteins of unknown function (Figure 3). From the entire annotated ES protein dataset, 24% (n=38) of proteins did not have any known function or known homologues in other organisms. Comparison of the results obtained for the three larval stages indicated an increase in the number of proteins involved in metabolic pathways from the L3-egg stage to the L3-lung and L4 stage larvae, whereas only two endochitinase homologues were identified from the L3-egg. In contrast, motor activity proteins, including proteins such as myosin-4, paramyosin and tropomyosin, were unique to L3-egg. Finally, 9% of proteins identified in L3-lung ES products, including cuticlin-1, cuticle collagen 12 and 13, represented 'structural' proteins, whereas those belonging to this category were less represented in L3-egg (none) and L4 (2%). Of the 17 binding proteins identified 82% of them were ATP-, ion-, carbohydrate- and DNA-binding proteins.

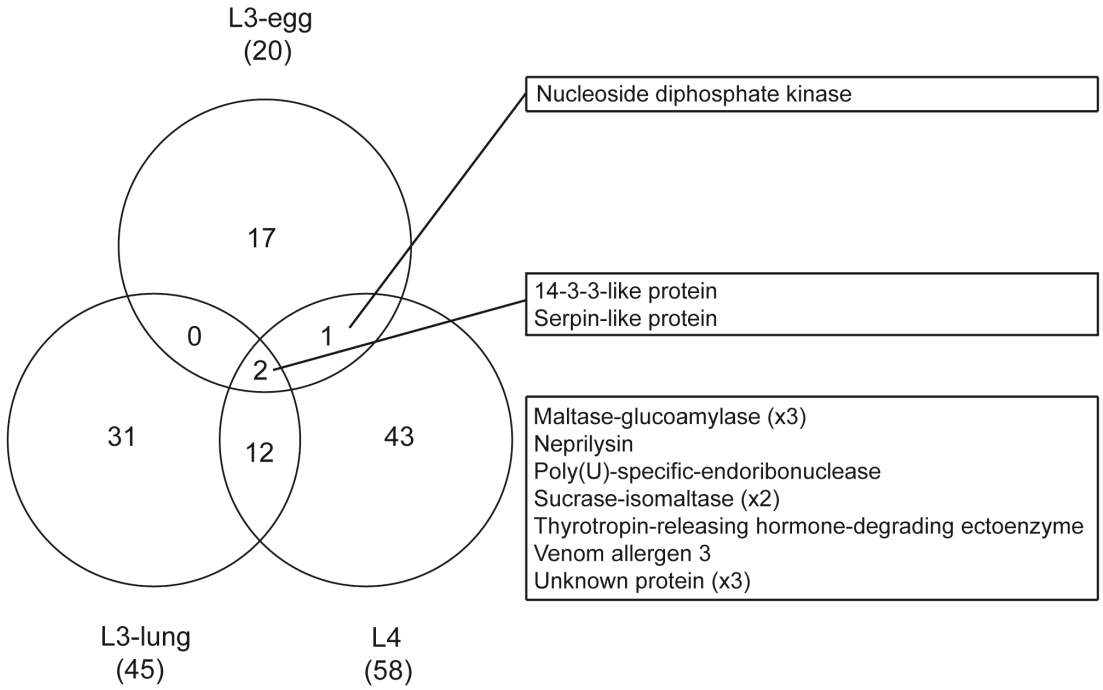

Figure 3.2: Venn diagram showing the distribution of the number of proteins identified in ES products from L3-egg, L3-lung and L4 of *A. suum*. The similar proteins identified are listed on the right.

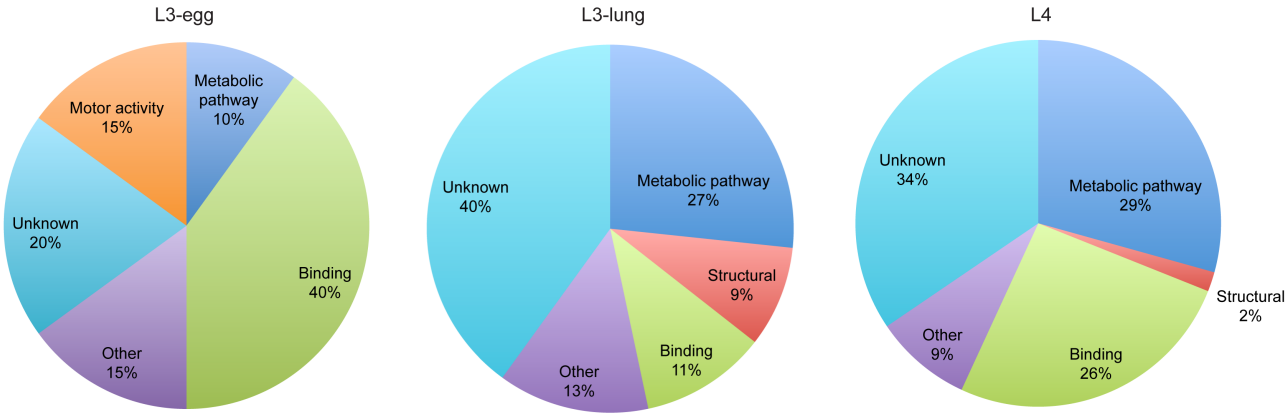

Figure 3.3: Gene Ontology terms relating to molecular function assigned to the proteins identified in ES products from L3-egg, L3-lung and L4 of *A. suum*.

Table 3.2: Protein identifications in *A. suum* L3-egg ES products

Category/Protein identity	ID[a]	Score	Coverage %	Unique sequences	emPAI	Sig[b]
Metabolic pathway						
Endochitinase	GS_04985	205	13	2	0.24	++
	GS_08584/ GS_15811[d]	146	18/ 10	1	0.57	++
Motor activity						
Myosin-4	GS_03209	54	1	1	0.02	-
Paramyosin	GS_12985	122	3	3	0.1	-
Tropomyosin	GS_20722	97	6	1	0.12	-
Binding						
14-3-3-like protein	GS_05590	174	14	4	0.51	-
32 kDa beta-galactoside-binding lectin lec-3	GS_06140	96	5	1	0.10	+
Globin-like protein	GS_08818	73	12	2	0.21	-
Histone H2A*	GS_13585	99	23	2	0.67	++
	GS_19480	141	25	2	0.67	++
Histone H2B.1/H2B.2	GS_12983	55	19	2	0.67	++
Histone H4	GS_02440	166	40	4	2.3	-
Nucleoside diphosphate kinase	GS_12864	98	6	1	0.33	-
Other						
Serpin-like protein	GS_15000	49	19	3	0.27	-
	GS_19115	50	7	2	0.09	-
	GS_19745	77	4	1	0.11	-
Unknown						
PAN domain-containing protein	GS_06520	265	12	4	0.27	+
Hypothetical protein CBG_20511	GS_15101	79	11	2	0.08	++
Unknown	GS_06759	125	8	3	0.17	++
Unknown	GS_19262	77	1	1	0.03	-

a The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). b The identified proteins were predicted to be either a classical secretory protein (+), non-classical secretory protein (++) or not secreted (-) by secretion prediction using SignalP and SecretomeP.

Table 3.3: Protein identifications in *A. suum* L3-lung ES products

Category/Protein identity	ID[a]	Score	Coverage %	Unique sequences	emPAI	Sig[b]
Metabolic pathway						
Maltase-glucoamylase	GS_07553	78	2	1	0.05	+
	GS_15893	142	6	2	0.15	-
	GS_16769	132	8	3	0.18	++
	GS_23879	171	4	4	0.08	++
Neprilysin-1	GS_08219	66	1	1	0.02	+
Sucrase-isomaltase	GS_01568	277	19	4	0.66	-
	GS_02444	258	13	3	0.28	-
	GS_05716	80	11	1	0.19	-
	GS_08447	69	2	1	0.04	++
	GS_17323	129	3	1	0.05	-
	GS_20796	121	2	1	0.04	-
	GS_22047	151	6	3	0.12	++
Structural						
Cuticlin-1	GS_10816	63	3	1	0.09	-
Cuticle collagen 12	GS_16238	147	17	2	0.27	++
Cuticle collagen 13	GS_12737	289	8	1	0.34	++
Peptidyl-prolyl cis-trans isomerase A	GS_15602	197	7	2	0.29	-
Binding						
14-3-3-like protein	GS_05590	73	3	1	0.11	-
C-type lectin	GS_12842	102	49	2	1.70	-
Latent-transforming growth factor β-binding protein 1	GS_21305	102	0	1	0.01	++
Thyrotropin-releasing hormone-degrading ectoenzyme	GS_02555	123	4	1	0.08	++
Transmembrane cell adhesion receptor mua-3	GS_11192	253	2	6	0.06	++
Other						
Aspartic protease 6	GS_13572	239	9	2	0.25	+
Pepsin inhibitor Dit33	GS_22518	81	6	1	0.13	++
Poly(U)-specific endoribonuclease	GS_22743	101	4	1	0.05	+
Protein DAO-2	GS_24324	120	13	1	0.54	+
Serine protease	GS_07735	78	2	1	0.05	+
Serpin-like protein	GS_19115	303	14	2	0.29	-
Unknown						
24 kDa protein of As22	GS_08591	219	17	2	0.36	+
DOMON domain-containing protein	GS_00339	144	20	2	0.43	-
Excretory/secretory mucin MUC-5	GS_22776	529	56	1	4.62	++
Heh-1	GS_20415	66	5	1	0.12	-
Transthyretin-like protein 5	GS_01881	85	9	1	0.25	+
Venom allergen 3	GS_10381	103	12	2	0.26	++
von Willebrand factor domain-containing protein	GS_02090	66	3	1	0.07	++
Hypothetical protein LOAG_00319	GS_14306	72	14	1	0.30	-
Hypothetical protein LOAG_07538	GS_11367	109	5	1	0.12	-
Unknown	GS_01811	72	14	1	0.29	-
Unknown	GS_02698	83	11	1	0.33	-
Unknown	GS_03310	89	4	1	0.14	+
Unknown	GS_09456	119	20	2	0.56	+
Unknown	GS_10718	171	34	2	0.92	+
Unknown	GS_12589	133	11	1	0.58	+
Unknown	GS_15853	245	18	2	0.67	-
Unknown	GS_17230	198	11	2	0.49	-
Unknown	L3E_00366	150	4	1	0.21	-

a The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). b The identified proteins were predicted to be either a classical secretory protein (+), non-classical secretory protein (++) or not secreted (-) by secretion prediction using SignalP and SecretomeP.

Table 3.4: Protein identifications in *A. suum* L4 ES products

Category/Protein identity[a]	ID[b]	Score	Coverage %	Unique sequences	emPAI	Sig[c]
Metabolic pathway						
Fructose-bisphosphate aldolase 1	GS_19276	189	14	3	0.28	-
Fumarate reductase	GS_20429	78	2	1	0.07	-
Glutathione S-transferase 1	GS_16802	150	11	1	0.16	-
Maltase-glucoamylase	GS_00984	65	5	1	0.13	++
	GS_07553	93	21	1	0.16	+
	GS_15893	642	32	9	1.00	-
	GS_18934	101	7	2	0.20	++
	GS_21210	66	15	1	0.43	++
	GS_23879	1143	16	20	0.46	++
Neprilysin-1	GS_08219	1114	14	14	0.39	+
	GS_10348	198	4	5	0.13	++
	GS_19140	331	6	6	0.14	++
Phosphoenolpyruvate carboxykinase GTP	GS_20378	63	4	1	0.08	-
Sucrase-isomaltase	GS_05716	175	25	3	0.67	-
	GS_08447	97	1	1	0.04	++
	GS_16354	95	5	1	0.08	++
	GS_19777	259	6	4	0.14	++
Structural						
Peptidyl-prolyl cis-trans isomerase 3	GS_07454	82	8	1	0.21	++
Binding						
14-3-3-like protein	GS_05590	96	3	1	0.11	-
Aminopeptidase N	GS_04166	106	4	3	0.13	++
	GS_05584	143	3	1	0.05	++
	GS_05746	696	14	12	0.29	+
C-type lectin protein 160	GS_02845	194	7	2	0.36	+
	GS_04559	835	35	8	1.76	+
	GS_12996	170	10	3	0.26	++
Enolase	GS_21295	87	3	1	0.08	-
GH family 25 lysozyme 2	GS_22190	441	39	5	3.27	++
Nucleoside diphosphate kinase	GS_12864	66	9	1	0.15	-
Phosphatidylethanolamine-binding protein	GS_22941	103	16	2	0.19	++
Thyrotropin-releasing hormone-degrading ectoenzyme	GS_02555	404	26	7	0.66	++
Zonadhesin	GS_01761	771	22	9	1.00	++
	GS_11354	66	1	1	0.04	+
	GS_11656	693	24	4	0.71	+
Other						
Aspartic protease 6	GS_14901	340	15	2	0.17	+
	GS_15316	753	24	5	0.90	+
	GS_19445	919	26	5	1.24	+
Poly(U)-specific endoribonuclease	GS_22743	638	16	7	0.56	+
Serpin-like protein	GS_19115	395	18	3	0.29	-
Unknown						
24 kDa protein [*A. simplex*]	GS_07900	68	16	1	0.41	++
As14	GS_02102	217	25	1	0.26	+
Transthyretin-like protein 5	GS_21838	69	18	1	0.40	-
Transthyretin-like protein 46	GS_02516	119	18	2	0.45	+
Venom allergen 3	GS_10381	131	16	3	0.41	++
Unknown	GS_01911	63	4	1	0.11	++
Unknown	GS_01916	63	6	1	0.20	+
Unknown	GS_01929	89	5	1	0.13	+
Unknown	GS_03310	264	13	2	0.67	+
Unknown	GS_03433	90	3	1	0.08	-
Unknown	GS_04618	125	5	1	0.28	-
Unknown	GS_06231	183	20	3	0.74	+
Unknown	GS_08453	82	11	2	0.30	-

Unknown	GS_08951	613	14	6	0.38	++
Unknown	GS_09456	116	9	1	0.56	+
Unknown	GS_11305	123	1	1	0.03	++
Unknown	GS_23530	126	17	2	0.43	+
Unknown	L3E_00366	124	8	2	0.21	-
Unknown	L4_01560	131	32	2	0.95	++
Unknown	L4_03658	162	20	1	0.31	+

a The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). b The identified proteins were predicted to be either a classical secretory protein (+), non-classical secretory protein (++) or not secreted (-) by secretion prediction using SignalP and SecretomeP.

3.3.3 Glycosyl hydrolases in Ascaris suum

The most frequently identified proteins in ES products were glycosyl hydrolases belonging to family 31 (GH31). In total 16 GH31 proteins were identified in the ES products of L3-lung and L4 larvae with homology to maltase-glucoamylases and sucrase-isomaltases. Six and 5 GH31 proteins were identified in L3-lung and L4, respectively, and another 5 for both of these larval stages.

In order to obtain more information on these proteins, we subsequently BLAST searched the *A. suum* genome for additional members of this GH31 family. In total, 32 protein sequences were identified, all showing homology to GH31 proteins (Table 3.5). The length of the protein sequences ranged from 80 to 1772 amino acids (aa), suggesting that some of the sequences were not full length. Twenty of the predicted GH31 proteins were predicted as either secreted through a classical or non-classical pathway.

The GH31 protein sequences (≥ 700 aa) representing *Ascaris* were aligned with those of homologous proteins from other species for subsequent phylogenetic analysis (Figure 3.4, panel A). The unrooted tree indicated clustering of the majority of the GH31 proteins of *A. suum* with acid-active GH31 enzymes (i.e. AAG, SUIS, MGA, AAGR1-2), whereas only one (i.e. GS_18807) clustered with neutral-active GH31 enzymes (i.e. GANAB and GANC). The results of a comparative analysis of the amino acid sequence homology around the catalytic site of 13 *A. suum* GH31 proteins (codes GS_0471, GS_05082, GS_06701, GS_08447, GS_13054, GS_17123, GS_17323, GS_18807, GS_19777, GS_20796, GS_22047 and GS_23879) and the 4 GH31 proteins present in *C. elegans* (AAGR1-AAGR 4) (Figure 3.4, panel B) indicated that the signature motifs around the catalytic nucleophile are largely conserved between these two nematode species.

In the transcriptomic analysis, the RPKM values for all GH31 proteins identified here showed that most of them are transcriptionally upregulated in the late larval stages (L3-lung and L4) of *A. suum* (Table 3.5). Based on the RPKM values, GH31 proteins with the highest transcription were GS_18934, GS_13054 and GS_19777, with RPKM values of > 500 in L4. A qPCR analysis of genes encoding

GH31 proteins (codes GS_18934 and GS_19777) was conducted to (1) verify the transcriptomic data and (2) to analyse their transcription profiles in different tissues of adult *A. suum* (Figure 3.5, panel A). Indeed, transcription levels of both genes were higher in L4 compared with other stages. In addition, the transcription linked to these GH31 was in the intestine of both female and male adults of *A. suum*, whereas almost no transcription was detected in either the reproductive system or the cuticle of both sexes (Figure 3.5, panel A).

To confirm the intestinal location of the GH31 proteins, enzymatic assays were performed to measure glycolytic activity in protein homogenates from different adult *A. suum* tissues (Figure 3.5, panel B). Particularly maltose and sucrose were degraded following incubation with homogenates from the intestinal tracts of both adult male and female worms. The glycolytic activity measured was markedly higher in the water-insoluble protein fractions compared with the water-soluble fraction ($P<0.05$). In addition, the intestinal homogenates from males showed higher activity compared with females ($P<0.05$). The degradation of lactose was only observed after incubation with the water-insoluble protein fraction produced from the adult male intestines.

Table 3.5: List of glycosyl hydrolases identified in the A. suum genome, their sequence length and their gene levels in different larval stages

			RPKM values				
Gene ID[a]	**AA length**	**Sig[b]**	**L3-egg**	**L3-liver**	**L3-lung**	**L4**	**ES material[c]**
GS_05082	735	-	0,2	0,02	0,03	0,02	
GS_16769	587	++	0,06	13,45	0,2	1,14	+
GS_17323	600	+	0,33	141,38	11,02	9,61	+
GS_04731	1047	+	0,02	4,64	1,33	1,05	
GS_10423	226	++	29,27	435,4	182,9	11,19	
GS_00493	153	-	0,11	0,11	0,16	0,09	
GS_01568	311	-	0,05	0,05	0,08	0,05	+
GS_02444	380	-	0,04	0,04	0,06	0,04	+
GS_08026	107	++	0,15	0,15	0,22	0,13	
GS_08447	895	++	0,02	0,02	0,03	0,02	+
GS_16354	408	++	0,04	0,04	0,06	0,03	+
GS_23821	195	+	0,08	0,08	0,12	0,07	
GS_24300	573	++	0,03	1,3	2,52	0,05	
GS_07553	217	+	0,08	0,08	0,11	0,07	+
GS_15893	459	-	0,04	0,04	26,65	0,93	+
GS_22047	830	++	0,02	0,02	0,03	0,02	+
GS_00984	255	-	0,06	0,06	0,09	0,06	+
GS_20796	743	-	0,02	0,02	0,03	0,02	+
GS_21210	80	-	0,2	0,2	0,3	0,18	+
GS_18807	935	-	8,58	17,06	62,88	22,98	
GS_06701	828	+	2,07	2,87	50,98	39,96	
GS_18934	359	++	8,29	2,02	249,19	989,82	+
GS_05716	181	-	0,09	0,09	0,13	1,1	+
GS_13054	457	++	0,22	5,71	15,67	705,7	
GS_17123	737	++	0,02	0,02	0,03	0,48	
GS_21706	521	++	0,03	0,67	0,09	11,18	
GS_23879	1772	++	0,61	0,39	9,98	56,22	+
GS_04250	479	-	1,65	9,45	4,42	38,01	
GS_00096	460	++	0,04	0,11	0,05	97,35	
GS_12078	373	-	0,04	0,04	0,06	3,05	
GS_19777	966	++	1,07	24,87	159,14	1054,46	+
GS_23076	995	+	0,02	0,02	2,49	17,89	

a The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). b The identified proteins were predicted to be either a classical secretory protein (+), non-classical secretory protein (++) or not secreted (-) by secretion prediction using SignalP and SecretomeP. c. GH31 proteins identified in the ES material are marked with an '+'

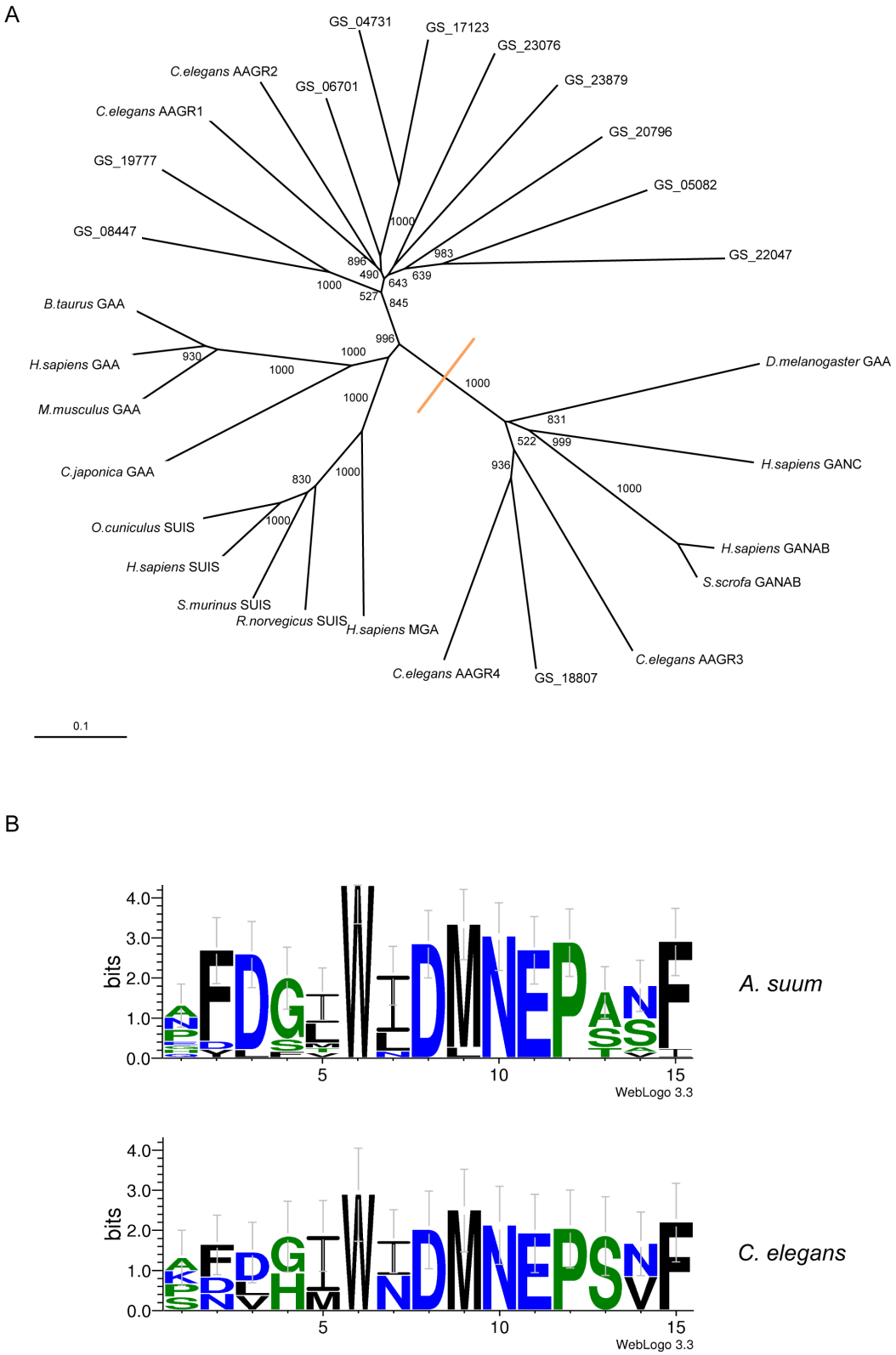

Figure 3.4: Phylogenetic tree and signature motifs of GH31 proteins A. Unrooted phylogenetic tree of the *A. suum* GH31 proteins (with a minimum sequence length of 700 amino acids) and other selected eukaryotic GH31 protein following neighbour-joining analysis. The values at the branch nodes represent bootstrap values (maximum 1000). B. Comparative analysis of the amino acids around the catalytic nucleophile (Trp and Asp) of GH31 proteins for the *C. elegans* GH31 proteins AAGR1-4 and the *A. suum* GH31 proteins (GS_04731, GS_05082, GS_06701, GS_08447, GS_13054, GS_17123, GS_17323, GS_18807, GS_19777, GS_20796, GS_21706, GS_22047 and GS_23879).

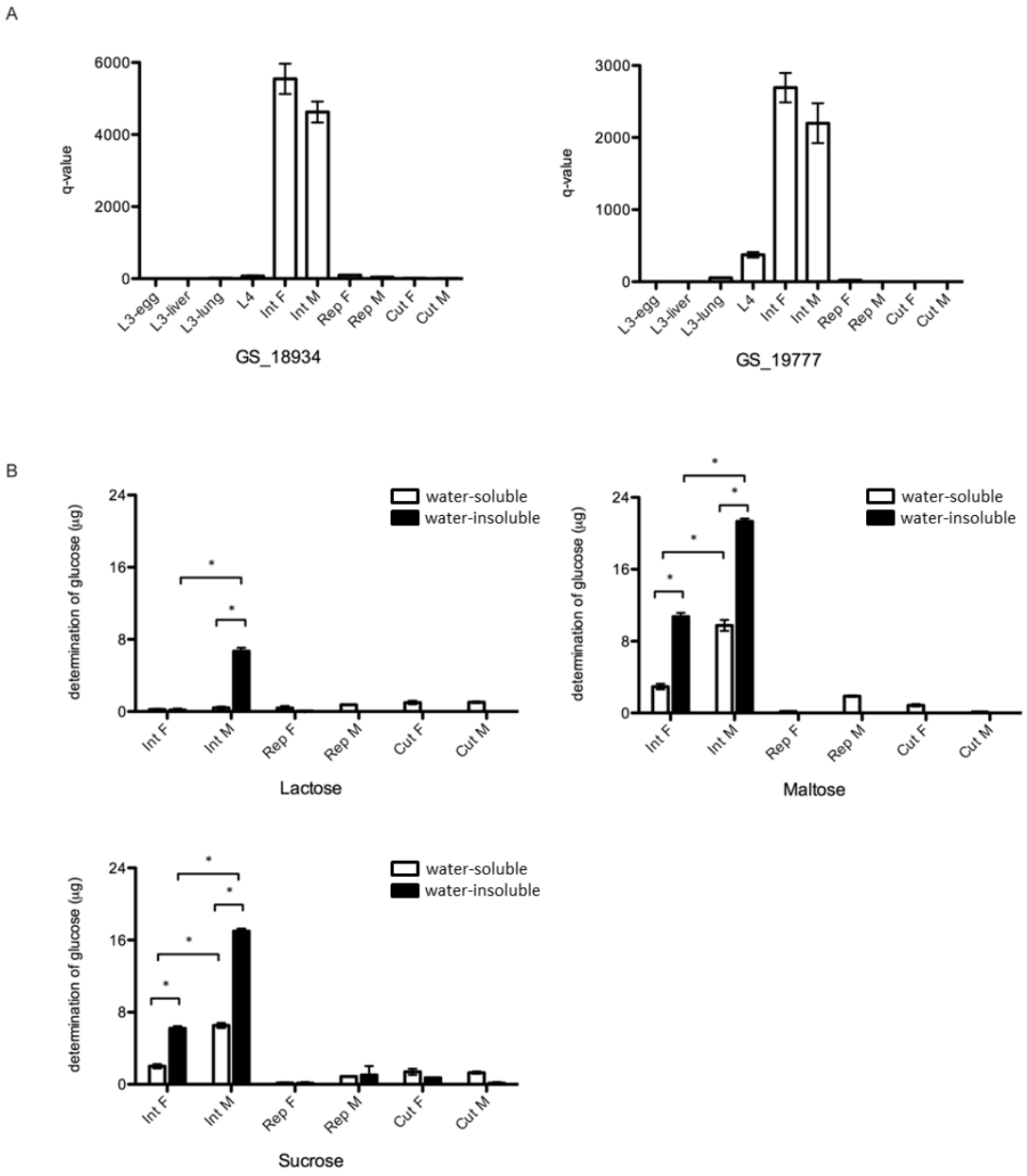

Figure 3.5: qPCR and glucosidase hydrolytic activity analysis of GH31 proteins. A. A qPCR analysis for two GH31 proteins on cDNA produced from different larval stages and adult worm tissues. B. Comparison of the glucosidase hydrolytic activity in water-soluble (white bars) and water-insoluble extract (black bars) from different adult tissues. Results are shown as average + SD. Substrates used in the assays were lactose, maltose and sucrose. Int F: female intestine; Int M: male intestine; Rep F: female reproductive system; Rep M: male reproductive system; Cut F: female cuticle; Cut M: male cuticle. (* $P < 0.05$)

3.4 Discussion

The goal of this chapter was to identify the ES proteins produced and released by the larval stages of *A. suum in vitro* and to infer the functions of these molecules during the migratory phase of the parasite through the body of the host animal. In total, 106 proteins were identified, of which 62% were predicted to either contain a signal peptide, suggesting secretion through a classical pathway, or predicted to be secreted *via* a non-classical pathway. The other 38% of proteins lacked a detectable signal sequence. Although no changes were observed in the motility or physical appearance of the larvae during the *in vitro* culture, some atypical secreted proteins were detected. The highest number of 'non-secreted' proteins for L3-egg was 55% compared with 45% and 26% for L3-lung and L4, respectively. Some of these 'atypical secreted' proteins, including 14-3-3 and serpin, may include their secretion in extracellular vesicles as described for other helminths, such as *C. elegans* [236], *Fasciola hepatica* and *Echinostoma caproni* [237]. However, the presence of some typical intracellular proteins in the ES material, such as histones, for example, suggests that there was some cellular damage in the larvae leading to leakage of intracellular proteins into the medium. The precise reason for this is unclear, but it is possible that the hatching procedure and the subsequent washing steps have a role. Moreover, keeping the *in vitro* culture as short as possible may help reducing the possibly invisible leakage of intracellular proteins into the medium. Therefore, in the future it would be interesting to analyse ES material that has been collected after only few hours of *in vitro* culture.

Among the 106 ES proteins identified in this chapter, two (i.e. a serpin-like and a 14-3-3 protein) were released by all three larval stages investigated. Serpins are serine protease inhibitors with a wide spectrum of functions in numerous biological systems, such as blood coagulation, complement activation and inflammation [223, 225, 238, 239]. Analysis of the *A. suum* genome and transcriptomes showed that they contain 10 serpin-encoding genes [151], whereas 8 and 3 serpin genes have been identified in the genomes of *C. elegans* and *B. malayi* [238], respectively. A number of studies have previously reported

on the presence of serpins in nematode ES products and experimental evidence indicates that many of them can have an immune-evasive function [239]. Interestingly, earlier studies of *A. suum* have shown that the activities of host proteases, such as trypsin and chymotrypsin, were greatly decreased from the micro-environment of live worms with a functioning gastrointestinal system [240]. Subsequently, Martzen *et al.* [241, 242] showed that inactive chymotrypsin complexes were formed in the muscle sarcolemma and in the epithelial surface of the gut of adult *A. suum* as well as in developing eggs and larvae of this nematode. In this way, the serine protease inhibitors may not only protect the worms from degradation in host digestive environment but might also mask the surface of developing larvae, permitting them to evade the host's immune system as they migrate from the intestine to the liver and the lungs. Whether the serpins detected in the ES products from *A. suum* are involved in these processes is still unclear.

In addition to the serpin, a 14-3-3 protein was also detected in the ES material of all three larval stages. Such 14-3-3 proteins represent a family of relatively conserved regulatory proteins, which can bind a range of functionally diverse signaling proteins. In *C. elegans*, a 14-3-3 protein regulates *daf-2*/insulin-like signaling pathway, which is critical for regulating development, longevity, metabolism and stress resistance [243]. Although the 14-3-3 proteins have been isolated and characterized recently as molecules with a significant role in the parasite biology and immunology within the context of the host–parasite relationship [244-246], currently, little information is available on their actual role in parasites.

Further comparison of the protein composition of the larval ES proteins showed that more overlap existed between L3-lung and L4 compared with L3-egg. Glycosyl hydrolases belonging to family 31 were particularly prominent in ES products from L3-lung and L4. The identification of 16 GH31 proteins is an intriguing outcome of this study, particularly since no other studies have reported the presence of such enzymes in the ES products from nematodes. An analysis of the *A. suum* genome and transcriptomes revealed 32 putative GH31

protein encoding genes/sequences. Although the exact number of GH31 protein genes in *A. suum* is less than 32, because of short or incomplete sequences in the current dataset, it is still clear that this gene family has undergone a large expansion compared with other nematode species. A preliminary analysis indicated the presence of only 4 GH31 protein genes in the genomes of *C. elegans*, *B. malayi* and *T. spiralis* (results not shown). The results presented in the present study also indicated that most of the GH31 proteins were transcriptionally upregulated from the L4 larval stage onwards, with a peak in the adult stage of *Ascaris*, in particular in intestinal tissues. This finding was also confirmed by enzymatic assays, showing the highest glycosidase activity in intestinal protein extracts from adult worms. It has been suggested [247] that *Ascaris* takes most of its nutrients from the partially digested host food in the intestine. The present findings suggest that the degradation of complex carbohydrates forms an essential part of the energy metabolism of this parasite once it is established in the small intestine. The highest level of glycolytic activity was consistently found in the water insoluble protein fraction, suggesting that the enzymes are associated or directly bound to a cell membrane. In mammals, it is well established that disaccharidases, such as the sucrose-isomaltase complex, are bound to the apical membrane of the gut-epithelial cells, for example through a highly hydrophobic segment in the N-terminal region of the protein [248, 249]. However, none of the *A. suum* GH31 protein sequences were predicted to contain such a transmembranic region. Therefore, further research is needed to determine the exact cellular location of the GH31 proteins within the intestinal tissues of *A. suum*. If the results would show that they are actually located on the intestinal surface, it would make them interesting drug and/or vaccine targets.

Apart from the shared proteins, most of the proteins identified were unique to a particular larval stage. Amongst the ES proteins identified in L3-egg, there were at least two different endochitinases. Chitinases are enzymes that catalyze the hydrolysis of beta-1, 4-*N*-acetyl-d-glucosamine linkages in chitin polymers. Studies of *B. malayi* (a filarioid nematode) showed that a chitinase was secreted during the exsheathment process of the microfilariae in the mosquito vector and from the eggshell during hatching of the larvae within the reproductive tract of

the adult stage [185]. RNAi studies of *A. viteae* (also a filarioid) showed that chitinase was also critical in the moulting process of the nematode [186]. Interestingly, Geng *et al.* [184] previously reported on the abundant secretion of a chitinase in the perivitelline fluid surrounding the infective *A. suum* larva just prior to hatching from the egg. This chitinase is however different from that identified here. During the larval cultivation, many of the larvae need to lose the L2 cuticle, which is usually still present around the infective L3 larvae when they hatch from the egg. Therefore, it is possible that the chitinases identified herein are involved in the exsheathment process.

Analysis of ES products from L3-lung and L4-ES resulted in the identification of various proteins that have consistently been found in ES material of other parasites, such as the transthyretin-like proteins, C-type lectins and venom allergens [221, 223, 224, 250]. The transthyretin-like proteins are one of the largest conserved nematode-specific protein families of which the function is still largely unclear. Recent data published by Wang *et al.* [251] on TTR-52, one of the 57 transthyretin-like proteins present in *C. elegans* suggest that these proteins act extracellulary to mediate cell-cell interactions.

C-type lectins belong to a type of carbohydrate-binding protein family, known as lectins. These molecules are widely distributed throughout the animal kingdom and have a diverse range of functions, including cell-cell adhesion, immune responses to pathogens and apoptosis [221, 252]. Notably, C-type lectins were also particularly abundant in the secretions from *T. canis* and hookworms [250, 253]. The recent report of the sequence similarity of C-type lectins from *A. suum* to host to dendritic cell receptors suggests that the parasites may utilize lectins to bind to carbohydrate moieties on the surface of host cells to avoid pathogen recognition mechanisms in hosts [200]. The identification of several C-type lectins in the current study could indeed indicate that they play an important, yet undiscovered, role at the parasite-host interface.

Venom allergens belong to the SCP/TAPS protein family and are basically found in every species investigated so far. Despite the fact that the exact function of

SCP/TAPS proteins remains unknown, various studies have shown that they are amongst the most abundant proteins expressed and secreted during the transition from the free-living to the parasitic life stages, suggesting an important role in the onset of parasitism [175]. Compared with the number of SCP/TAPS proteins identified in the ES material of some other species [176-178], it is surprising that only 2 were identified in the current study. However, this finding is concordant with previous evidence from genomic and transcriptomic datasets [151] indicating the presence of only 12 SCP/TAPS-encoding genes in the *A. suum* with relatively limited transcription levels (results not shown).

In conclusion, this study provides the first in-depth characterization of the ES products from the larval stages of *A. suum*, a crucial step in enhancing our knowledge and understanding of the biology of this parasite and its interactions with its mammalian host. In order to characterize the other group of exposed proteins, i.e. the cuticle surface associated proteins, the next chapter will focus on the identification of these molecules and evaluation of two surface protein identification approaches.

CHAPTER FOUR

Proteomic analysis of the cuticle surface associated proteins from the infective stage larvae of *Ascaris suum*

Based on:
Wang T., Van Steendam K., Dhaenens M., Vlaminck J., Deforce D., Jex A., Gasser R.B., Geldhof P.. Evaluation of a biotinylation labeling and an enzymatic shaving approach to identify cuticle surface associated proteins from the infective stage larvae of *Ascaris suum*. In preparation

4.1 Introduction

Ascaris, like other nematodes, possess a biologically unique surface. The dominant feature is the cuticle, a complex extracellular structure which is composed primarily of protein with trace amounts of lipid and carbohydrate [163]. The basic structure of the cuticle from outside to inside consists of the epicuticle, the external cortical region, the medial and basal layers [163]. The molecules expressed at the cuticle surface, together with ES proteins, which can be released from the cuticle surface [214, 215], represent the primary host-parasite interface [215]. They are thought to be the first signals perceived by the host and therefore play a critical role in the induction and development of immune responses [254-256]. Nematodes molt four times in their life cycle, each time replacing their existing cuticle with the formation of a new cuticle. Therefore, different stages of a single species may express different proteins on their cuticular surface as part of an immune evasion strategy. For example, host antibody and leukocyte attachment to *Toxocara* larvae stimulates rapid shedding of the surface coat, leaving behind an abandoned glycocalyx to which host cells remain attached [215, 257].

Two technical approaches have previously been described to identify surface associated proteins of helminths. Braschi and Wilson biotinylated live *Schistosome* worms and subsequently enriched and identified the labeled proteins by tandem mass-spectrometry [233]. A similar approach was also applied on *A. suum* larvae and resulted in the labeling of 19 different proteins from the L3-egg and L3-lung stages [258], but these were not further characterized. In addition to biotinylation, an enzymatic shaving approach using trypsin has also been used to identify the surface proteins of worms. By using this approach, Pérez-Sánchez *et al.* [259] identified the tegumental proteomes of male and female *S. bovis* worms, in particular the proteins expressed on the outermost layers of the tegument structure. Later, the same method was employed to study the newly excysted juveniles of *F. hepatica*, resulting 40 tegument surface associated proteins [260]. In addition, Marcilla *et al.* [261] identified 13 surface associated proteins of *Strongyloides stercoralis* third stage

larvae by using this approach.

The aim of this study was to identify the surface associated proteins of the infective stage larvae of *A. suum* by applying both the biotinylation and enzymatic shaving approach followed by tandem mass-spectrometry.

4.2 Materials and Methods

4.2.1 Parasite material

Adult worms of *A. suum* were collected from naturally infected pigs at the local slaughterhouse. Eggs of *A. suum* were obtained from the uteri of female worms, and cultured in 0.1% $K_2Cr_2O_7$ for 28-30 days at 25°C. After 90% of the eggs had become fully embryonated, the infective stage L3 larvae were hatched from the eggs as described previously by Urban and Douvres [152] and then separated from eggshell fragments and other debris by baermannization by washing in PBS.

4.2.2 Identification of surface associated proteins through biotinylation

For labeling with sulfo-NHS-biotin (Pierce), the larvae were resuspended in PBS containing 0.5mg/ml of sulfo-NHS-biotin for 10 min at room temperature followed by three washes with PBS. After biotinylation, labeled and unlabeled control larvae were collected and incubated with streptavidin-FITC (Sigma) in the dark at 22°C for 15 min. Following the incubation, the larvae were washed three times with PBS, and then put on a glass slide for fluorescence microscopy analysis.

Protein extracts of both labeled and unlabeled control larvae were produced by grinding the frozen material to a fine powder in a liquid nitrogen-cooled pestle and mortar. The powder was sequentially subjected to a two-step process with reagents of increasing solubilising power [233]. For the water-soluble protein fraction, 4 ml of PBS, pH 7.4, were used to resuspend the powder for 2 h at 4°C by gentle 'head-over-head' mixing. The insoluble material was pelleted by

centrifugation at 120,000 *×g* for 15 min and the supernatant retained. For the water-insoluble protein fraction, the pellet was incubated at 22°C for 3 h using an extraction buffer consisting of 5 M urea (Sigma), 2 M thiourea (Sigma), 2% CHAPS (Sigma) and 2% SB3-10 (Sigma) in 40 mM Tris, pH 7.4. The supernatant was collected, as described for the water-insoluble protein fraction. A cocktail of protease inhibitors (Sigma) was added to each extract to avoid proteolytic degradation. Protein concentrations were measured with the Bradford reagent (Sigma), and proteins stored at -80°C.

The biotinylated proteins in each of the two extracts were subsequently isolated by affinity chromatography. A column with 250 µl gel volume of streptavidin-agarose beads (Sigma) was prepared according to the manufacturer's recommendations (ÄKTAexplorer™, GE Healthcare). Extracts were applied to pass over the affinity column at a speed of 0.1ml/min. After extensively washing the column with 4M Urea containing 0.1% SDS, the bound molecules were recovered from the streptavidin beads by adding 250 µl of 5% SDS and heating at 90°C for 10 min. The beads were pelleted and the supernatant was removed for further analysis.

The presence of biotinylated proteins in the purified material was evaluated by Western blotting using a streptavidin-HRP conjugate (Bethyl laboratories). Five µg of the purified extracts were fractionated on 12% reducing SDS-PAGE gels according to standard procedures [228] and subsequently blot transferred onto PVDF membranes (Millipore). The membranes were blocked in PBS containing 0.05% Tween 80 (PBST), and probed with streptavidin conjugated to horseradish peroxidase (diluted 1:120,000 in PBST) for 1 hour. Signals were visualized with the ECL detection system (Amersham). In addition to the Western blot analysis, 20 µg of the purified protein extracts were also separated on 12% SDS-PAGE gels and subsequently stained with Coomassie Brilliant Blue (Invitrogen), the entire lanes of interest were horizontally sliced in 10 equal pieces for subsequent liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis.

Tryptic in-gel digestion was performed as previously described [229]. In brief, to ensure better transfer of buffers, each protein band was cut into 1 mm^2 portions, washed twice in 50% acetonitrile with 25 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol in 25 mM ammonium bicarbonate, alkylated with 100 mM iodoacetamide in 25 mM ammonium bicarbonate and digested with trypsin (200 ng per band) at 37°C for 18 h. Peptides were extracted with acetonitrile and dried in a Speedvac. The whole biotinylation labeling process was repeated once.

4.2.3 Identification of surface associated proteins by enzymatic shaving

A schematic overview of the technical approach followed for the enzymatic shaving is shown in Figure 4.1. The purified larvae, approximately 300000 per sample, were gently washed three times with 50 mM NH_4HCO_3 and subsequently incubated with 500 µl digestion buffer (5 µg/ml trypsin contains 1mM DTT in 50 mM NH_4HCO_3) in 37°C for different times (i.e. 1, 3, 5, 10, 20, 40, 60 and 90 min) during which they were gently shaken. Control samples were setup for each time point in an identical way but without the addition of trypsin. Following the incubation, the samples were centrifuged and the larvae separated from the supernatants. The larvae were used to microscopically check their physical appearance. The supernatant samples from the trypsin treated larvae were further incubated for 16 h at 37°C to allow a full trypsin digestion of the proteins. For the control samples, the same concentrations of trypsin and DTT were added and the samples incubated for 16 h at 37°C.

After the incubation, all samples were dried in a speedvac and the peptides dissolved in 40 µl 0.1% formic acid (FA). Twenty µl was desalted for 10 min on a C-18 pre-column (C18 PepMap100, 5 µm x 5 mm, i.d. 300 µm Dionex) with 0.1% FA. Separation was performed by means of reversed phase nano-HPLC (25cm PepMap C18 analytical column, Dionex) at 60°C using a linear gradient of H_2O: ACN (97:3, 0.1% FA) to H_2O: ACN (20:80, 0.1% FA) at 300 nl/min over 70 min. The different peptides were analyzed on an ESI Q-TOF Premier (Waters, Wilmslow) in a data dependent mode, with automatic switching between MS and MS/MS for up to 7 higher charge ions, when the intensity of the individual ions rose above 50 counts per sec. Fragmentation of the precursors was performed

by means of CID. The capillary voltage was set at 1.9 kV, and the cone voltage was set at 100. M/z ratios for MS ranged between x and y and for MS/MS between x and y. M/z ratios selected for MS/MS were excluded for 150 sec. A custom collision energy profile was used.

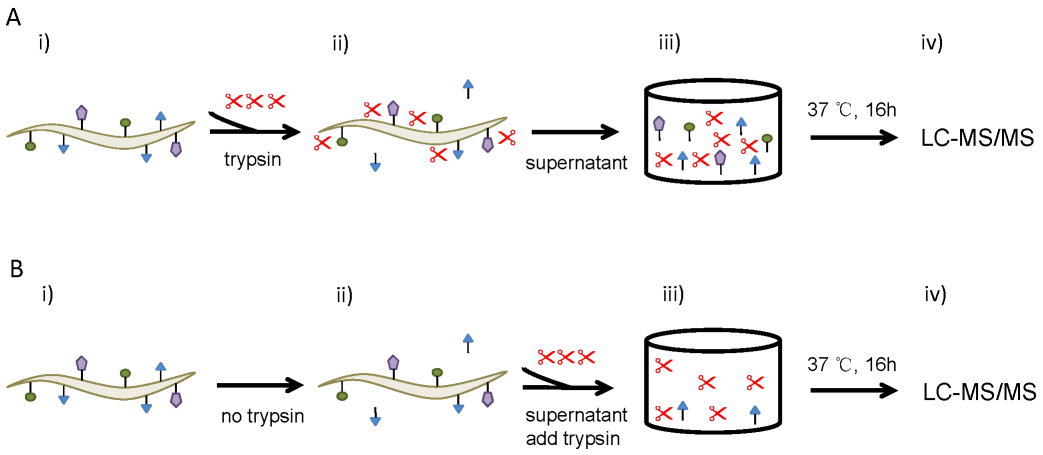

Figure 4.1: Approach for the identification of surface proteins in *A. suum* larvae. A: Larvae shaving: (i) fresh larvae were gently washed; (ii) incubation buffer containing trypsin and DTT was added to larvae for different time courses (1, 3, 5, 10, 20, 40, 60 and 90 min); (iii) supernatant contains shaved surface exposed peptides were collected; (iv) after 16 h incubation at 37°C, the supernatant were subjected to LC-MS/MS. B: Trypsin free control: (i) fresh larvae were gently washed; (ii) incubation buffer without trypsin and DTT for different time courses (1, 3, 5, 10, 20, 40, 60 and 90 min); (iii) supernatant contains released peptides were collected and digested with trypsin; (iv) after 16 h incubation at 37°C, the supernatant were subjected to LC-MS/MS.

4.2.4 Database searching and sequence analysis

Data were searched against an in-house *Ascaris* sequence database (18,542 protein entries), which is based on the recently published *A. suum* genome [151], using the search engine Mascot Daemon (v.2.3, Matrix Science, London, UK), allowing a maximum of one miscleavage. Carbamidomethyl (C) was specified as fixed modification and carbamidomethyl (N-term), deamidated (NQ) and oxidation (M) were considered as variable modifications for in-gel digest. For in solution digests, methylthio (C) was selected as the fixed modification, and deamidated (NQ) and oxidation (M) as variable modifications. An error-tolerant Mascot search was performed as well. The peptide tolerance and MS/MS tolerance were set to 0.35 Da and 0.45 Da, respectively. Only the most parsimonious group of protein identifications were reported from the identified proteins, and the identification threshold was set at $p < 0.01$. For the proteins

that were annotated based on only one peptide, the identification threshold was set at p <0.0001. An estimate of the relative abundance of the predicted proteins in the trypsin digestion was assessed using the Exponentially Modified Protein Abundance Index (emPAI) [231] together with the MS score, sequence coverage, detected peptides numbers. For redundant identifications, the emPAI value from the hit with the highest score was considered. The Gene Ontology (GO) database was used for inferring the molecular function of individual proteins identified. The protein sequences were analysed for the presence of signal peptides regions with SignalP 3.0. The subcellular localization was predicted with SecretomeP 2.0.

4.3 Results

4.3.1 Identification of surface associated proteins through biotinylation

Microscopical analysis of the biotinylated larvae following incubation in FITC labeled streptavidine showed a strong fluorescent signal on the surface of the worms, whereas no fluorescence was observed on the non-biotinylated larvae incubated in FITC labeled streptavidin (Figure 4.2).

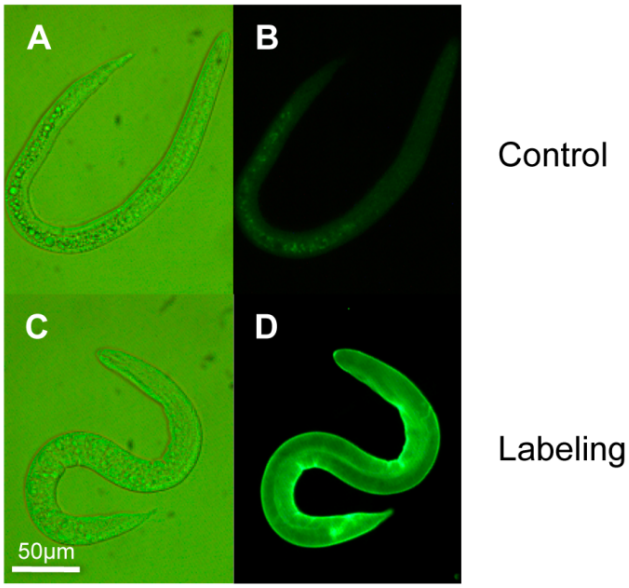

Figure 4.2: Microscopical analysis of the biotinylated- and control *A. suum* larvae stained by streptavidin-FITC. A and C: transmitted light images; B and D: UV light images.

The labeled proteins were extracted and then purified with streptavidin-agarose beads and finally analyzed on SDS-PAGE followed by Coomassie blue staining. In

the water-soluble protein fraction, many sharp bands were visible in the samples from the labeled worms compare to control worms (Figure 4.3, panel A). Most biotin labeled proteins bands were distributed between 37–50 kDa with some few fainter bands visible in the 75-100 kDa range. In the water-insoluble protein fraction, SDS-PAGE analysis of the labeled proteins revealed a complex banding pattern with most proteins ranging from 40 and 150 kDa. In the control samples of both the water-soluble and -insoluble protein fractions, only one band could be clearly observed of around 75 kDa.

To verify that the proteins recovered from the streptavidin-agarose beads were biotinylated, the purified proteins were separated by SDS-PAGE, blotted onto a PVDF membrane and then probed with streptavidin-HRP. In addition to the protein bands visualized by Coomassie blue staining, a large number of additional bands were detected by Western blotting, especially in the low molecular weight region (Figure 4.3, panel B). A number of additional bands were also observed in the control samples of both the water soluble and –insoluble fraction.

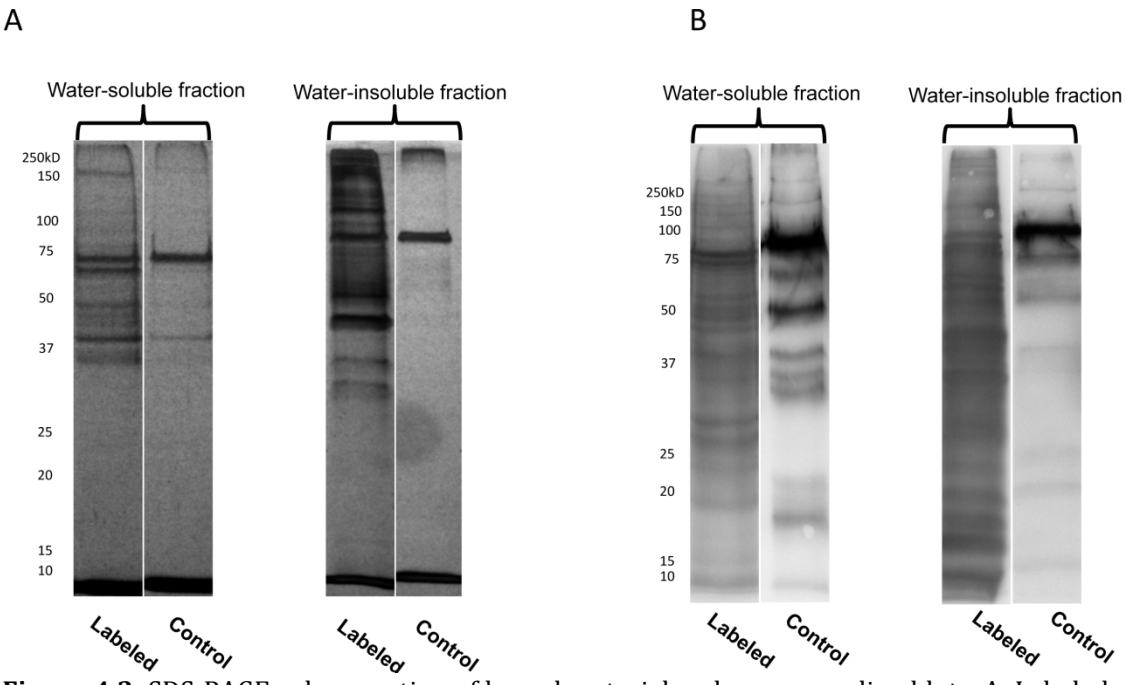

Figure 4.3: SDS-PAGE gel separation of bound material and corresponding blots. A: Labeled and control water-(in)soluble fractions (water-soluble protein fraction: PBS, pH 7.4; water-insoluble protein fraction: 5 M urea, 2 M thiourea, 2% CHAPS and 2% SB3-10 in 40 mM Tris, pH 7.4) recovered by streptavidin-agarose beads, separated by SDS-PAGE and stained by coomassie blue. B: corresponding blots probed with strep-HRP.

In a next phase, the entire SDS-PAGE gel lanes of both the water-soluble and –insoluble protein fractions from both labeled and non-labeled worms were horizontally sliced in 10 equal pieces for LC-MS/MS analysis in order to identify the proteins present in each fraction. The whole experiment, i.e. labeling procedure, protein extraction and purification and LC-MS/MS analysis, was performed twice and only the proteins uniquely identified in the protein extracts from labeled worms in the 2 independent experiments were regarded as putative surface associated proteins. This approach resulted in the identification of 17 putative surface proteins, of which 5 were present in the water soluble fraction (adenylate kinase isoenzyme, 14-3-3 like protein, tubulin alpha chain, heat shock protein 70A and peroxiredoxin), 11 in the water insoluble fraction (cytochrome b-C1 complex subunit 2, 2 vitellogenins, lamin-1, myosin-3, paramyosin, troponin I 2, ATP synthase subunit alpha and beta, flavoprotein subunit of succinate dehydrogenase and microtubule-actin cross-linking factor 1) and 1 protein (myosin) that was detected in both protein fractions (Table 4.1). The proteins identified were subsequently categorized by their molecular function according to the information obtained from the Gene Ontology database, including metabolic pathways (n=4), structural (n=1), motor activity (n=4), binding (n=6) and stress response (n=2). As indicated in Table 1, myosin, paramyosin, vitellogenin and lamin were, based on the MS information of score, sequence coverage, number of detected peptides and emPAI value, relatively highly abundant in the purified protein fractions.

Table 4.1: The surface associated proteins identified from the infective stage larvae of *A. suum*

Category/Protein identity[a]	ID[b]	Score	Coverage %	Unique sequences	emPAI	fraction [c]
Metabolic pathway						
Adenylate kinase isoenyzme	GS_11140	210	10%	1	0.4	WS
Cytochrome b-c1 complex subunit 2	GS_13329	101	2%	1	0.07	WI
Vitellogenin-6	GS_19373	554	9%	11	0.24	WI
	GS_10956	330	6%	7	0.14	WI
Structural						
Lamin-1	GS_16160	829	14%	6	0.52	WI
Motor activity						
Myosin-3	GS_09583	365	7%	9	0.17	WI
Myosin-4	GS_03209	1160	17%	19	0.43	WS, WI
Paramyosin	GS_12985	741	19%	12	0.65	WI
Troponin I 2	GS_05122					WI
Binding						
14-3-3-like protein	GS_05590	225	8%	2	0.36	WS
ATP synthase subunit alpha	GS_01787	400	6%	3	0.31	WI
ATP synthase subunit beta	GS_17447	343	17%	6	0.54	WI
Flavoprotein subunit of succinate dehydrogenase	GS_06887	402	15%	2	0.64	WI
Microtubule-actin cross-linking factor 1	GS_12069	546	2%	10	0.09	WI
Tubulin alpha chain	GS_14905	157	7%	2	0.14	WS
Stress response						
Heat shock protein 70 A	GS_10592	92	14%	1	0.41	WS
Peroxiredoxin	GS_21096	85	17%	2	0.39	WS

a The proteins identified were categorized by their molecular function according to information obtained from the Gene Ontology database. b The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). c Gel fraction: water-soluble fraction(WS); water-insoluble fraction (WI).

4.3.2 Identification of surface associated proteins by enzymatic shaving

After the trypsin treatment for different periods of time (ranging from 1 up to 90 minutes), the larvae were microscopically examined to investigate their physical appearance. The larvae incubated with trypsin for up to 20 min displayed no morphological differences compared to the untreated worms. However, with the extension of the incubation times (40, 60 and 90 min), the larvae started to appear damaged and flaccid, suggesting poor conservation of cuticle integrity and an excessive penetration of the trypsin. Therefore, only the samples incubated for maximum 20 min were further used for protein identification by LC-MS/MS.

Mascot searches of the MS/MS spectra resulted in the identification of 22 proteins that were present in the control samples, of which 15 proteins were identified at least 2 time points (summarized in Table 4.2). A similar approach resulted in the identification of 22 proteins in the samples collected from the trypsin treated larvae, of which 16 were detected at different time points (Table 4.2). Thirteen out of these 16 proteins were also detected in the control samples, whereas 3 were unique for the trypsin-treated larvae. All identified proteins could be subdivided into 6 different GO function groups: metabolic pathway, structural, motor activity, binding, stress response and proteins of unknown function (Table 4.2). The top three most abundant GO terms are binding, metabolic pathway and stress response. *In silico* prediction of classical and non-classical secretion showed that 7 proteins were predicted to be either a classical or non-classical secreted protein (Table 4.2).

Table 4.2: The surface asscociated and released proteins identified from the infective stage larvae of *A. suum*

Category/Protein identity[a]	ID[b]	Score	Coverage %	Unique sequences	emPAI	Shaving time point min	Control time point min	Sig.[c]
Metabolic pathway								
Adenylate kinase isoenzyme	GS_11140	69	17	2	0.40	5,20	1,20	-
Fructose-1,6-bisphosphatase isozyme 2	GS_03621	103	5	1	0.10	5	1,20	-
Glyceraldehyde-3-phosphate dehydrogenase	GS_20130	199	8	2	0.22	1,3,5	1,3,5,10,20	++
Phosphoenolpyruvate carboxykinase GTP	GS_04959	383	26	3	0.47	5,20	1,3,5,20	-
Structural								
Actin-2	GS_02399	188	8	3	0.24	5,10,20	1,3,5.10,20	+
Peptidyl-prolyl cis-trans isomerase 3	GS_07454	72	8	1	0.21	None	1,20	++
Cuticle collagen lon-3 [d]	GS_00376	155	8	1	0.11	3,5,10,20	None	
Motor activity								
Myosin-4	GS_03209	137	1	2	0.04	5,20	1,20	-
Binding								
12 kDa FK506-binding protein	GS_05903	79	12	1	0.34	10	3,5,10	++
14-3-3-like protein	GS_05590	210	17	5	0.68	5,20	1,3,5,10,20	-
Enolase	GS_21295	290	17	5	0.58	5	1,3,20	-
Histone H2B 2	GS_05263	54	19	2	0.66	None	1,3	++
Tubulin alpha chain	GS_16262	126	3	2	0.13	5,10,20	None	
Stress response								
Heat shock protein 70	GS_03797	85	2	1	0.05	5,20	1,20	-
Heat shock protein 90	GS_17449	142	3	2	0.09	5	1,3,20	-
Small heat shock protein OV25-1	GS_01974	104	13	2	0.42	3,5,10,20	1,3,5,10,20	++
Small heat shock protein OV25-1	GS_02287	191	16	2	0.43	3,5,10,20	1,3,5,10,20	++
Unknown								
Unknown	GS_19262	111	2	2	0.06	5,20	None	

a The proteins identified were categorized by their molecular function according to information obtained from the Gene Ontology database. b The accession number in *A. suum* genome database (available on WormBase, www.wormbase.org). c The identified ES proteins were predicted to be either a classical secretory protein (+) and non-classical secretory protein (++) or not secreted (-) by secretion prediction using SignalP and SecretomeP. d The identified surface assoicated proteins were underlined.

4.4 Discussion

In this study, we employed two approaches to explore the surface proteome of *Ascaris* larvae.

The methodology of the biotin labeling approach, as previously described [258, 262-264], was modified on several aspects. First, in an attempt to minimize the labeling of non-surface associated proteins that are potentially released by the worms during the labeling procedure itself, the biotin labeling reaction time was shortened to 10 minutes compared to the 30 minutes standardly used in previous studies. Second, a protein extract produced from non-biotin labelled *Ascaris* larvae was processed and analysed in exactly the same way as the labelled samples in order to distinguish naturally biotinylated proteins that potentially exist in *Ascaris* from the potential surface-associated proteins that were labelled by the applied procedure. The proteins identified in these control samples (n=17) were subtracted from the list of proteins identified in the biotinylated samples. Third, since the Western blot analysis with streptavidin indicated the presence of a large number of additional protein bands that were not visible by Coomassie blue staining, we decided to submit the whole gel lanes for LC-MS/MS analysis. Finally, to avoid the contamination of non-labelled proteins that are potentially pulled down by protein-protein interactions, the beads were slowly and extensively rinsed with a 4M Urea 0.1% SDS buffer as an extra step before boiling the streptavidin beads to release the labeled proteins. The use of this modified approach resulted in the identification of 17 putative surface associated proteins, 5 of which were present in the water soluble fraction, 11 in the water insoluble fraction and 1 that was detected in both protein fractions. Among the total 17 identifications, cytochrome, peroxiredoxin, 14-3-3, myosin, paramyosin, and tubulin have been suggested as surface proteins in other helminths. Apart from these proteins, most of the identified proteins are predicted to be intracellular. Whether their identification is caused by the presence of damaged worms in the cultures is still unclear. Previous studies on trematodes described the removal of damaged worms from the cultures after microscopic analysis [233, 265]. This approach is unfortunately

not feasible with the infective stage *A. suum* larvae used in this particular study due to their small size (approximately 250 μm).

As summarized above, the trypsin shaving approach has recently been used on several helminth species as a faster and gel-free alternative for the biotin labeling approach to identify surface associated proteins. In addition, the shaveome approach also has the advantage that the analysis can be performed on small amounts of biological material, for example nematodes derived from host tissues. The approach followed in the current study differs from the previously applied methods on two aspects. First, the incubation time with the trypsin was optimized in order to avoid tissue damage in the worms, which would automatically lead to the release of cytoplasmic proteins in the medium. Our results indicated that 5 minutes incubation with trypsin was optimal as 89% of all detected proteins were already identified in these samples while the larvae still appeared undamaged. Second, trypsin-free control samples were also included in our experiment in order to distinguish excretory-secretory proteins from the genuine surface associated/bound proteins. These control samples consisted of larvae that were incubated in buffer only for the same period of time as the trypsin treated larvae. The use of this modified approach resulted in the identification of 3 putative surface associated/bound proteins, i.e. a cuticle collagen, a tubulin and a protein with unknown function. Among them, tubulin has already been found as a surface compound in *Schistosoma japonicum* [264] and *S. bovis* [259]. Concerning the unknown protein (GS_19262), this protein was also shown in the ES analysis of the L3 egg stage (see Chapter 3 table 3.2), suggesting that this protein could also just be released. LC/MS-MS analysis of the control samples resulted in the identification of 15 proteins that are released by the larvae during this short *in vitro* culture. Some of these proteins are likely to be actively secreted whereas others are potentially released from the surface of the worms.

Comparison of tables 4.1 and 4.2 shows that only 4 proteins were identified by both methodologies. This is not completely surprising as the methodologies used are based on totally different principles. The biotin labeling approach is based on

the presence of an exposed primary amine group in the protein, either at a lysine residue or at the N terminus of the protein [266, 267], whereas the trypsin shaving approach requires the presence of a trypsin cleavage site in a part of the protein that is accessible to trypsin. The proteins identified by both methodologies as being surface-associated and/or released by the larvae are adenylate kinase isoenzyme, tubulin alpha chain, heat shock protein 70, myosin-4 and 14-3-3-like protein. Interestingly, myosin-4 and the 14-3-3 like protein were also detected in the ES material of the third stage larvae cultured *in vitro* for 5 days (see Chapter 3 table 3.2).

Myosins represent a family of ATP-dependent motor proteins with critical roles in a wide range of actin-based motility processes, including muscle contraction [268]. Myosin is often found in association with paramyosin [38] which is present in a diverse group of invertebrate species [268]. Previous studies of *Ascaris* myosin and paramyosin were mainly focused on their roles in muscle layer [269-271], but no reports were yet made of their presence on the surface or in ES products of *Ascaris* larvae. Several other studies however have suggested or shown the surface localization of these two motor proteins in several helminth species [259, 261, 272, 273]. Furthermore, it was suggested that paramyosin serves not only as a structural protein but can also act as an immunomodulatory agent [273]. For example, the paramyosin of *T. spiralis* and *S. mansoni* could strongly bind human complement components C8 and C9 and protect the helminths from being attacked by host complement [272, 274, 275]. Based on all this, it would be interesting to further unravel the role of these proteins in *A. suum* larvae.

The 14-3-3-like proteins are well known as kinase and phosphatase. In *C. elegans*, a 14-3-3 protein regulates *daf-2*/insulin-like signaling pathway, which is critical for regulating development, longevity, metabolism and stress resistance [243]. However, there are also several reports describing that 14-3-3-like proteins are involved in reactive oxygen species (ROS) regulation and apoptosis in parasitic nematodes [276, 277]. Parasitic nematodes, like all aerobic organisms, require antioxidant enzymes to cope with ROS generated during

cellular metabolism [278]. Besides, they have to protect themselves against ROS produced by the host. Therefore, the regulation and evasion of ROS is important for parasitic nematode survival [278, 279]. In the present study, in addition to 14-3-3-like proteins, a set of anti-ROS molecules were revealed, including peroxiredoxin (PRX) by the biotine labelling approach and GAPDH as a released protein in the shaving experiment. PRX is produced by a wide range of organisms in defense against toxic hydroxyl radicals that damage proteins, lipids and DNA [198]. Various parasites are known to express PRX on the surface during the infection process to cope with ROS [198], including *Globodera rostochiensis* [280], *F. gigantica* [281]*, S. japonicum*[282, 283] and *Taenia solium* [284]. The expression of PRX on the surface in the infective stage *Ascaris* larvae might provide a barrier between the parasites and host, protecting the worms from host ROS. The potential ROS scavenger GAPDH is a glycolytic enzyme, but it has been suggested that the presence of GAPDH on the surface of schistosomes may help to protect them from oxygen-mediated attack by phagocytes [285]. The presence of these anti-ROSs in the exposed proteins might indicate their putative important role as an antioxidative protector for *A. suum* at the host-parasite interface.

The surface proteins of nematodes potentially play an important role in the infection process and survival of the parasites *in vivo* and could therefore be potential drug or vaccine targets. Here we have investigated the surface associated and released proteins of infective stage *A. suum* larvae by a biotin labeling and an enzymatic shaving approach. This resulted in the identification of several interesting proteins that warrant further research in order to fully unravel their role in parasite biology.

CHAPTER FIVE

General discussion and conclusions

The objective of this doctoral thesis was to investigate the basic biology of *A. suum* larvae and to identify molecules that potentially play essential roles in parasite survival and development during the hepato-tracheal migration. In this final chapter, we will first discuss and evaluate the technical approaches used in this thesis. In addition, based on the outcome of the transcriptomic and proteomic analyses, a shortlist will be presented of some of the genes and proteins identified that are believed to be key players in fundamental biological processes of the parasite and that are therefore worthwhile further investigating as potential drug- or vaccine targets against *Ascaris*.

5.1 Identification of *Ascaris suum* excretory-secretory proteins

Excretory/secretory proteins likely play a critical role at the host-parasite interface. Classical gel-based ES proteomic investigations have been performed in many other parasitic nematodes including *A. caninum*, *B. malayi, H. contortus*, *T. circumcincta* and *T. spiralis* [176, 221-227]. All these proteomic studies were based on a well-established approach of producing and retrieving parasite ES material by culturing the parasites *in vitro* and collecting the culture medium either daily or at the end of the culture period, which can be up to 5 days. Current experiments we identified 106 different proteins in the ES material of *A. suum*, 20 in L3-egg, 45 in L3-lung and 58 in L4 stage. Some of these molecules were already known to be involved in the parasite-host interface, however, some typical intracellular proteins, such as histones, were also present in the ES material, suggesting that there might be some intracellular contents leaking into the medium through cellular damage. The presence of such intracellular proteins in nematode ES material is commonly observed [221, 286-288]. It is therefore hard to believe that this is caused by dying or dead worms, especially since most studies control for parasite viability and integrity. The ES products of these organisms basically consist of a mixture of both actively and passively released proteins, the latter coming from the gut and surface of these metabolically active worms. Furthermore, non-classical excretion/secretion of proteins is also recognized to be common in parasites.

5.2 Identification of surface associated proteins of *Ascaris* larvae

The outcome of the biotin labeling- and trypsin shaving approach in chapter 4 was rather surprising. First, very little proteins were identified by both approaches and second, most of the proteins identified are supposed to be located intracellularly. Since non-classical excretion/secretion of proteins in nematodes has been described, it is still possible that some of these proteins are actually presented on the worm surface. A way to confirm this would be to produce recombinant version of some of these proteins and subsequently generate polyclonal antibodies against them in rabbits. These antibodies could then be used to analyse and potentially confirm the surface localization of the proteins analysed. This approach is however time consuming,

5.3 Potential *Ascaris* drug- and vaccine targets identified by the proteomic and transcriptomic analyses

5.3.1 Glycosyl hydrolases

From the in-depth transcriptome analysis performed in chapter 2, we found that the carbohydrate metabolism becomes increasingly important for the larvae during their development, in particular when they establish in the small intestine. At transcript level, genes involved in general carbohydrate metabolism accounted for approximately 4% of the total transcriptome in the L3-egg stage larvae. This proportion further increased to 6% in the L3-liver and L3-lung stages and almost 10% in the L4 larvae. Most intriguingly, the group of genes involved in starch and sucrose metabolism was upregulated by over 11 times in the intestinal stage L4 larvae compared to the infective stage L3 larvae (Figure 5.1). In line with this transcriptome analysis, the proteomic analysis of the excretory-secretory material of these larval stages in chapter 3 showed the high abundance of glycosyl hydrolases (maltase-glucoamylases and sucrose-isomaltases) from the L4 stage onwards. Additional qRT-PCRs and enzymatic assays showed that these glycosyl hydrolases were mainly transcribed and expressed in the intestinal tissue of the adult worms, further supporting the

hypothesis that they are important for the nutrition of the worms.

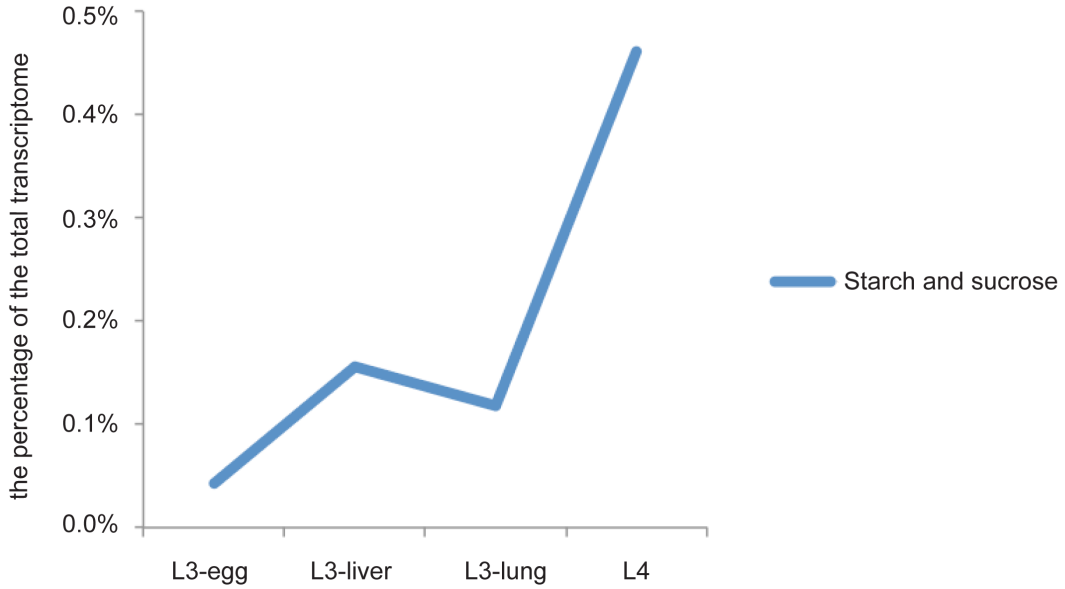

Figure 5.1: The ratio of the total RPKM values of all the genes involved in starch and sucrose metabolism compared to the total transcription.

Sucrose-isomaltase (digests branched starch linkages) and maltase-glucoamylase (digests linear regions at the last step of starch hydrolysis) are two glycosyl hydrolases with complementary activities in the mammalian intestine [293-295]. The two enzymes are paralogs and belong to the glycosyl hydrolases family 31 (GH31) [296]. In *A. suum*, there are 32 genes homologous to family GH31 of *C. elegans*. Although the exact number of genes of GH31 proteins in *A. suum* is probably less than 32, due to the possible incomplete sequences in the current dataset, this gene family has clearly undergone a large expansion compared with other nematode species (e.g. only 4 GH31 protein genes in the genomes of *C. elegans*, *B. malayi* and *T. spiralis*). What is more interesting, 20 out of 32 GH31 proteins were predicted to be either a classical or non-classical secreted protein by *in silico* prediction, and 16 out of 32 GH31 proteins were indeed abundantly detected in the ES products of *A. suum* larvae at L3-lung and L4 stages, whereas no such enzymes were reported to be present in the ES products of other nematodes to date.

With the information that *A. suum* GH31 proteins are highly abundant in the ES products, i.e. at the important host-parasite interface, and that the GH31 family

likely plays an indispensable role in energy metabolism, we assume that a drug or vaccine targeting these glycosidases GH3 could be an efficient way to control this parasite. In further research, it would be interesting to determine the exact cellular location of the GH31 molecules within the intestinal tissues of *A. suum*. If they are actually located on the intestinal surface, as we presume, it would make it theoretically feasible to administrate the specific drug by simply adding it to the host food. However, several aspects should be taken into account with this type of approach. First, proteins belonging to a protein family often show considerable functional redundancy, so it is questionable whether a drug or vaccine targeting a single protein would actually be sufficiently effective. Second, since GH31 proteins are relatively highly conserved between organisms, the potential risk exists that a drug or vaccine would also target the enzymes of the host. Therefore, it still requires a considerable number of studies before any efforts are taken in developing a drug or vaccine targeting these proteins. Apart from analyzing their cellular localization, as described above, it would also be very meaningful to identify their substrate specificity in order to determine whether each of these enzymes in the worm degrades its own carbohydrate substrate.

5.3.2 C-type lectin

In chapter 2, we found three C-type lectins (GS_04559, GS_09853 and GS_17824) that were specifically transcribed in L4 larval stage, with RPKM values of 1697, 627 and 141 respectively. In addition, the one with the highest RPKM value, i.e. GS_04559, was also detected to be abundantly present in the ES material of the L4 stage larvae (chapter 3). The C-type lectin superfamily has emerged as one of the largest and most widely expressed set of proteins in the whole animal kingdom [221, 252]. They are characterized by a conserved 115-130 amino acid carbohydrate-recognition domain, which includes multiple Ca^{2+} binding sites. Many members of this superfamily are important in innate immune defenses against infection, such as antigen uptake and presentation, cell-cell adhesion, apoptosis and T cell polarization [199].

In the free-living nematode *C. elegans*, more than 270 genes encode C-type

lectins at the genomic level [289]. Many of them, such as *clec-50*, have been demonstrated experimentally to be upregulated and protective upon pathogen exposure, i.e. bacterial infection [19, 290-294]. Moreover, three C-type lectin genes (*clec-17*, *clec-60*, and *clec-86*) have a defense function against *Microbacterium nematophilum* [290]. In other nematodes, C-type lectin genes were observed to be primarily expressed in the gut-dwelling adult stages of *H. polygyrus* and *N. brasiliensis* [295], suggesting their potential antibacterial role. The detection of abundant expression of C-type lectins in the L4 larval stage (chapter 2) also raises the possibility that gut-dwelling nematodes utilize C-type lectins proteins to counter resident bacterial populations, in a manner analogous to the antibacterial defense functions of homologues in *C. elegans*.

Nevertheless, C-type lectins may fulfill not solely a defensive role within the nematodes, but a broader one in regulating interactions with other organisms (pathogens, symbionts, prey or hosts). For instance, a C-type lectin was expressed on the surface of the marine nematode *Laxusoneistus* and binds symbiotic bacteria necessary for the metabolism of this worm. C-type lectins were believed to interact with the host as well, since they were particularly abundant in the secretions of *T. canis* [250, 253] and hookworms [221]. These C-type lectins, for instance TES-32 and TES-70 in *T. canis*, showed greater homology to mammalian proteins such as CD23 (low affinity IgE receptor) and macrophage mannose receptor, than to any *C. elegans* protein [250]. Thus, it has been hypothesized that these secreted C-type lectins function to subvert the host immune response by binding to specific cell-surface carbohydrate moieties in parasitic nematodes [250, 253]. Moreover, TES-70 is able to bind mammalian carbohydrates in a calcium-dependent manner [253], suggesting a role in immune evasion by either inhibiting the migration of host cells or binding to and masking worm carbohydrates from host immune cells. The recent report of similarity between host dendritic cell receptors and C-type lectins from *A. suum* suggests that *Ascaris* may utilize lectins to bind to carbohydrate moieties on the host cells to avoid pathogen recognition mechanisms in hosts [200]. Finally, apart from immune evasion, a putative role in nematode sperm-egg recognition and fertilization was also suggested by a study on a non-secretory C-type lectin

in *A. ceylanicum* [296].

Therefore, taking all these potentially important functions of these proteins into account, C-type lectins could be interesting targets for *Ascaris* control. However, there are still a few biological aspects of these proteins that need to be further determined before any effort should be taken in developing a vaccine based on these proteins. First, previous studies showed that the C-type lectin genes respond in a pathogen-specific manner in *C. elegans* [289]. Most of them specifically expressed for one pathogen species, only few genes are upregulated by more than two pathogen species (*clec-63*, *clec-65*, *clec-67* or *clec-87*) [289]. In *C. elegans* alone, there are over 270 potential C-type lectin gene products, whereas only 15 potential C-type lectin genes were found in the *A. suum* genome. Thus, it is very likely that the regulation of C-type lectins as a defense against bacterial infection is different between *C. elegans* and *A. suum*. One C-type lectin gene should respond multiple pathogen species in *A. suum*. It should be extremely interesting to find out which genes are specific against which pathogen species *in vitro*. Second, although it is likely that secreted lectins play an essential role in the biology of parasitic nematodes, no experimental data exists to determine the origin of the secreted lectins. In the future, it will be important to distinguish between products released through the nematode digestive tract, and those from specialized excretory/secretory organs. Notably, by ELISA and Western blot Harcus *et al.* [295] found that polyclonal antibodies raised to C-type lectin of *N. brasiliensis* and *H. polygyrus* not only recognized secreted C-type lectin of these two gut-dwelling nematodes, but also bound strongly to the cuticle of adult *H. polygyrus*, raising the possibility that these products interfere in some manner with immunological recognition or effector function. However, no C-type lectin was identified in our analysis of the surface associated protein in chapter 4. This might be a result from technical limitations, which will be discussed later in this chapter.

5.3.3 14-3-3 protein

In chapter 3, a 14-3-3 protein (GS_05590) was found to be present in the ES products from the three different *A. suum* larval stages (L3-egg, L3-lung and L4)

investigated. Interestingly, this secreted 14-3-3 protein was also identified as a surface associated protein of the L3-egg stage by the biotin labeling approach in chapter 4. The 14-3-3 proteins are highly conserved ~30 kDa acidic dimeric proteins in all eukaryotes. They function in cell signaling, cell cycle regulation, intracellular trafficking, and other processes by modulating protein-protein interactions [297]. There are two isoforms of the 14-3-3 protein in *C. elegans*, *ftt-2* and *par-5*, but only *ftt-2* is involved in regulation of *daf-2*/insulin-like signaling pathway, which is critical for regulating development, longevity, metabolism and stress resistance [243].

It is proposed by most studies that 14-3-3 proteins are involved in the transmission of regulatory signals associated with cell physiology such as proliferation, migration and morphological changes during the parasite life cycle. 14-3-3 proteins have also been identified in a growing number of helminths, both nematodes and trematodes. In nematodes, such as *S. stercoralis*, *H. contortus*, *N. americanus, T. circumcincta*, the actual function of 14-3-3 proteins are still unclear [226, 277, 298]. However, in two trematodes species, i.e. *Schistosoma* and *Echinococcus*, more comprehensive investigations have been performed on multiple isoforms as well as sub-isoforms of the 14-3-3 proteins [246, 277]. The 14-3-3 proteins of trematodes were found located in the tegument or actively secreted. In *S. mansoni*, all three isoforms of 14-3-3 are expressed during infection and induced antibody production by the host [244]. Vaccination with the 14-3-3ζ isoform of *schistosome* resulted in parasite load reductions ranging from 25% to 65% [299-302]. The greatest success of using 14-3-3 as an anti-parasite vaccine antigen was achieved in *E. multilocularis*, where vaccination with the 14-3-3ζ isoform elicited worm reduction of even as high as 97% in mice [245, 246]. All these findings suggest that it is worthwhile to further evaluate these proteins as vaccine targets in *Ascaris* as well.

5.3.4 Transthyretin-like protein

A significant number of transthyretin-like protein (TLP) encoding genes were observed in the list of most abundant transcripts of all the larval stages (chapter 2). The transthyretin-like (*ttl*) gene family is one of the largest conserved

nematode-specific gene families, coding for a group of proteins with significant sequence similarity to transthyretins and transthyretin-related proteins [303]. TLPs have been identified in a large number of parasitic nematodes of plants, animals and human, including *Meloidogyne incognita*, *Radophilis similis* [303, 304], *B. malayi* [224], *O. ostertagi* [305, 306], *Trichostrongylus colubriformis* [307] and *H. contortus* [308]. In chapter 3, TLPs were also found in the ES products of *A. suum* L3-lung and L4. To date, no surface localization of nematode TLPs had been reported. The exact biological role(s) of the TLPs in nematode is still unclear. However, a yet uncharacterized role of TLPs in the nematode nervous system has been hypothesized, based on the experimental localized *ttl* encoding protein to the ventral nerve of *R. similis* (i.e. Rs-*ttl*-2) and to the nervous system and hypodermis of *C. elegans* (gene code R13A5.6; http://www.wormbase.org) [309]. Recent data published by Wang *et al.* [251] on TTR-52, one of the 57 TLPs present in *C. elegans* suggest that these proteins act extracellulary to mediate cell-cell interactions. In addition, two previous proteomic studies of *H. contortus* [225] and *A. caninum* [221] propose that TLPs could play a central role in the parasite-host interplay. In the next phase, in order to provide new insights in the biological role of these proteins, more information regarding the transcription profile(s) and tissue distribution of the TLPs in *Ascaris* should be obtained as well as research into the potential molecular ligands of these proteins.

5.4 Conclusions

The transcriptomic and proteomic investigations performed in this thesis have led to a significant progress in our understanding of *Ascaris* biology. The most important observations were the high levels of carbohydrate degrading enzymes in the developing larvae and the identification of several additional proteins that are likely essential for parasite survival and development. These observations provide a broad basis for further research into the molecular function of these proteins with the ultimate goal to determine whether they could be used as drug- or vaccine targets against this important parasite.

SUMMARY

Summary

The gastro-intestinal nematodes *Ascaris lumbricoides* and *Ascaris suum* are amongst the most prevalent parasites of humans and pigs, respectively. *Ascaris* infections cause serious public health problems and significant economic losses in the pig industry. Traditionally ascariasis is controlled by mass treatment with anthelmintics. However, due to the short activity of the anthelmintics and an environment often highly contaminated with *Ascaris* eggs, reinfections can occur rapidly. In addition, the development of anthelmintic resistance which has been observed in other nematodes, suggests that current repeated doses of massive chemotherapy treatment will probably also lead to drug resistance in *Ascaris* spp. eventually. Therefore, investigation of the alternative means of ascariasis control such as vaccination is worthwhile for pursuing. To promote the rational development of an effective vaccine, a better understanding of the molecular biology and host-parasite relationships of *Ascaris* is required.

In chapter two, a transcriptome dataset was generated and analyzed in order to identify both the highest transcribed and stage-specific transcripts for each larval stage, the metabolic changes and chemosensation pathways active in the larvae and, finally, the expression of potential molecular mimicry candidates. Illumina RNA sequencing of the *A. suum* infective-stage, liver-stage, lung-stage and intestinal-stage larvae resulted in 95,463,423 sequences yielding 18,543 contigs. Within the top 250 most highly transcribed contigs per stage, accounting for approximately 60% of the total transcription in each stage, cuticle collagens were by far the most abundant gene family. The analysis also identified a set of interesting stage-specific genes, such as venom allergen, chitinase, thioredoxin and cecropin, with a potentially important role in the host-parasite interaction processes. Analysis of the metabolic pathways showed that the degradation of complex carbohydrates likely forms an essential part of the energy metabolism of this parasite, as almost 10% of the total transcriptome of the L4 stage encodes for enzymes involved in this pathway. In comparison to *Caenorhabditis elegans*, a reduced number of olfactory molecules were identified in *A. suum*, in particular the aerotaxis molecules seem to absent, suggesting that they are less important for this parasite. Finally, 12 transcript sequences were identified as potential

molecular mimicry candidates, including a suppressor of cytokine signaling family. Overall, the outcome of this transcriptomic analysis provides novel and valuable information regarding the biology of *A. suum* larvae, which can be used as a foundation for further research.

In chapter three, we identified the excretory-secretory proteins of the migratory stages of *A. suum* utilizing LC-MS/MS. In total 106 proteins were identified, some of which are known as important players in the parasite-host interface. Interestingly, an abundance of glycosyl hydrolases was observed in the ES material of the intestinal L4 stage larvae. By combining the proteomic analysis with in-depth genomic, transcriptomic and enzymatic analyses we could show that the glycosyl hydrolase protein family has undergone a massive expansion in *A. suum* and that most of the glycolytic activity is present in the intestinal tissue of the adult parasites. Again, as already indicated by the transcriptomic analysis, this could suggest that the degradation of complex carbohydrates forms an essential part of the energy metabolism of this parasite once it establishes in the small intestine.

In chapter four, we employed two different approaches, i.e. biotin labeling and enzymatic shaving, combined with LC-MS/MS to study the cuticle surface associated proteins of the infective stage larvae of *A. suum*. In total, 17 proteins were identified as surface associated proteins by the labeling approach. Many of these molecules had been previously reported as surface exposed proteins in other helminth species. On the other hand, the MS/MS spectra for the shaving approach only resulted in the identification of 3 genuine surface attached proteins, i.e. a cuticle collagen, a tubulin and a protein with unknown function. These results extended the knowledge on the biology of *Ascaris* larvae and their cuticle proteins, which play important roles in host-parasite interactions and hopefully it will benefit the development of novel intervention strategies.

In conclusion, the transcriptomic and proteomic investigations performed in this thesis have led to a significant progress in our understanding of *Ascaris* biology. The most important observations were the potentially important role of

carbohydrate metabolism in the larvae during their hepato-tracheal migration and the identification of the molecules essential to parasite survival and development. The study provides a basis for further molecular investigations aimed at exploring the biological role of the proteins identified and their potential as vaccine and/or therapeutic targets.

Samenvatting

De gastro-intestinale nematoden *Ascaris lumbricoides* en *Ascaris suum* behoren tot de meest voorkomende parasieten van respectievelijk de mens en het varken. *Ascaris* infecties veroorzaken ernstige problemen voor de volksgezondheid en aanzienlijke economische verliezen in de varkenssector. Traditioneel wordt ascariasis gecontroleerd door behandelingen met anthelmintica. Ten gevolge van de korte activiteit van deze anthelmintica en de vaak sterk besmette omgeving, kan herbesmetting snel optreden. Bovendien werd de ontwikkeling van resistentie tegen ontwormingsproducten reeds in andere nematoden waargenomen. Dit suggereert dat het blijvend gebruik van herhaaldelijke dosissen anthelmintica uiteindelijk ook zou kunnen leiden tot de ontwikkeling van resistentie in *Ascaris* spp. Hierdoor is het noodzakelijk om alternatieve manieren voor de controle van ascariasis, zoals vaccinatie, verder te onderzoeken. Om de ontwikkeling van een effectief vaccin te bevorderen is echter een goede kennis vereist van zowel de moleculaire biologie als de gastheer-parasiet interacties van deze parasiet.

In hoofdstuk twee werd het transcriptoom gegenereerd van de verschillende larvale stadia van *Ascaris* (infectieve L3, Lever-stadium L3, long-stadium L3 en darm-stadium L4 larven). In deze dataset werd gezocht naar transcripten die het meest werden geproduceerd of specifiek werden geproduceerd in de verschillende larvale stadia. Veranderingen in de metabolische processen of chemosensorische pathways en de expressie van mogelijke kandidaten van moleculaire mimicry werden ook onderzocht. De Illumina RNA-sequencing van de verschillende larvale stadia van *A. suum* resulteerde in 95,463,423 sequenties die samen 18.543 contigs uitmaakten. Binnen de top 250 meest getranscribeerde contigs per stadium, goed voor ongeveer 60% van de totale transcriptie in elk stadium, waren cuticula collagenen verreweg de meest voorkomende gen-familie. De analyse identificeerde ook een aantal interessante stadium-specifieke genen met een potentieel belangrijke rol in de gastheer-parasiet interacties zoals het venom-allergen, chitinase, thioredoxine en cecropine. Uit de analyse bleek ook dat de afbraak van complexe koolhydraten waarschijnlijk een essentieel onderdeel vormt van het energiemetabolisme van deze parasiet aangezien bijna

10% van het totale transcriptoom van het L4 stadium voor enzymen codeert die betrokken zijn bij deze processen. In vergelijking met *Caenorhabditis elegans*, werden minder olfactorische moleculen geïdentificeerd in *A. suum*. Met name de aerotaxis moleculen lijken afwezig, wat suggereert dat deze minder belangrijk zijn voor *Ascaris*. Tenslotte werden 12 transcripten geïdentificeerd als potentiële moleculaire mimicry kandidaten, waaronder een suppressor van cytokine signalisatie. De resultaten van deze transcriptoom analyse schenken ons nieuwe en waardevolle informatie over de biologie van *Ascaris* larven. Dit kan als basis dienen voor verder bio-moleculair onderzoek.

In hoofdstuk drie werden de excretie-secretie (ES) eiwitten van de migrerende larvaire stadia van *A. suum* geïdentificeerd aan de hand van LC-MS/MS. In totaal werden 106 eiwitten geïdentificeerd, waarvan sommige bekend staan als belangrijke componenten in de interactie tussen gastheer en parasiet. In het ES materiaal van de intestinale L4 larven werd een overvloed van glycosylhydrolasen waargenomen. Aan de hand van eiwit-analyses, gecombineerd met genomische, transcriptomische en enzymatische data kon worden aangetoond dat de glycosylhydrolase eiwitfamilie sterk aanwezig is in *A. suum* en dat deze eiwitten zich voornamelijk bevinden in het darmweefsel van de volwassen parasieten. Zoals de transcriptoomanalyse reeds aangaf kan dit suggereren dat de afbraak van complexe koolhydraten een essentieel onderdeel van het energiemetabolisme van deze parasiet vormt vanaf deze zich in de dunne darm gaat vestigen.

In hoofdstuk vier werden twee verschillende methodes gebruikt om eiwitten te bestuderen die geassocieerd zijn met het oppervlak van de cuticula van de infectieuze larven van *A. suum*. Namelijk, het biotinyleren en het enzymatisch 'afscheren' van deze eiwitten. De eiwitten werden geïdentificeerd aan de hand van LC-MS/MS. In totaal werden 17 eiwitten als oppervlakte-geassocieerde eiwitten geïdentificeerd door de biotine-labelling methode. Veel van deze moleculen werden reeds eerder als oppervlak-geassocieerde eiwitten beschreven in andere wormsoorten. De LC-MS/MS analyses van de enzymatisch afgeschoren eiwitten konden enkel 3 echte oppervlak-geassocieerde eiwitten

identificeren, namelijk een cuticula collageen, een tubuline en een eiwit met onbekende functie. De resultaten bekomen in dit hoofdstuk helpen onze kennis over de biologie van *Ascaris* larven en hun oppervlakte-eiwitten verder uit te breiden en ondersteunen zo de mogelijke ontwikkeling van nieuwe interventiestrategieën.

De transcriptomische en proteomische onderzoeken uitgevoerd in dit proefschrift hebben geleid tot een significante vooruitgang in onze kennis van de biologie van *Ascaris*. De belangrijkste bevindingen waren de potentieel belangrijke rol van koolhydraat metabolisme in de larven tijdens hun hepato-tracheale migratie en de identificatie van verschillende moleculen die essentieel blijken te zijn voor het overleven en de ontwikkeling van deze parasiet. De resultaten van dit onderzoek vormen een basis voor verdere moleculaire onderzoeken die gericht zijn op het onderzoeken van de biologische rol van de geïdentificeerde eiwitten en hun potentieel als vaccin kandidaat en/of drugtarget.

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Yours sincerely,
Tao
汪涛

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