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**Secreted Proteins, Infectivity and  
Immunity to the Parasitic Nematode**  
*Nippostrongylus brasiliensis*

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## **Declaration of Originality**

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I hereby declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from published or unpublished work of others has been acknowledged in the text and appropriately referenced.

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## Abstract

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Comparative analysis of a recent isolate (J) and a laboratory-adapted strain (W) of the parasitic nematode *Nippostrongylus brasiliensis* found that the former had higher fecundity and gave rise to more persistent infections, although these traits were partially abolished after three years of laboratory passage, suggesting that infection dynamics can be modified by continuous high-dose propagation. Host immune responses to the two strains were similar in mode and magnitude. Proteins secreted by infective larvae (L3) and adult parasites showed some subtle differences between strains, although the activity of enzymes which might impact on persistence such as acetylcholinesterases and nucleotide metabolising enzymes were similar.

Activation of *N. brasiliensis* L3 was not influenced by host serum, but a 37°C temperature cue was sufficient to induce feeding and protein secretion. Rat skin extracts induced chemotaxis of L3 and also induced the secretion of pre-synthesised proteins, although feeding and subsequent protein secretion were unaffected. Analysis of L3 secreted products by two-dimensional immunoblotting revealed differential immune recognition of specific proteins. Analysis of host resistin-like molecules showed that they had no effect on parasite chemotaxis and feeding activities, in contrast to published data.

The venom-allergen homologue/ASP-like (VAL) proteins are important therapeutic targets found in all parasitic nematodes studied to date, and eight secreted variants of VALs have been discovered in *N. brasiliensis*. Although *N. brasiliensis* VALs (*NbVALs*) were found to be immunogenic during natural infection, immunisation with recombinant *NbVAL-7* did not protect mice against challenge. Moreover, natural infection induced antigen-specific IgE and Type I hypersensitivity reactions to *NbVALs*, suggesting that this may be an intrinsic property of these proteins which limits their use in immunoprophylaxis of nematode infection.

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## List of Abbreviations

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°C	Degree Celsius
<sup>125</sup> I	<sup>125</sup> Iodine
2-DE	Two-dimensional gel electrophoresis
<sup>35</sup> S-Met	<sup>35</sup> S-methionine
5'-NT	5'-nucleotidase
AAMΦ	Alternatively activated macrophages
Ab	Antibody
Abs	Absorbance
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen-presenting cell
APS	Ammonium persulfate
ASP	<i>Ancylostoma</i> secreted protein
ATP	Adenosine triphosphate
Aq	Aqueous
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
bp	Base pairs
C48/80	Compound 48/80
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CHAPS	(Cholamidopropyl)-3,3-dimethylammoniumpropylsulfate
cGMP	Cyclic guanosine monophosphate
cm	Centimetres
CO <sub>2</sub>	Carbon dioxide
ConA	Concanavalin A
cpm	Counts per minute
CR3	Complement receptor 3
CRISP	Cysteine-rich secretory protein
DAF	Dauer formation
DALY	Disability-adjusted life year
DB71	Direct blue 71
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EST	Expressed sequence tag
EtBr	Ethidium bromide
ExL3	Exsheathed L3
FBS / FCS	Foetal bovine serum / foetal calf serum
FcεR1	High-affinity IgE receptor
FITC	Fluorescein isothiocyanate
g	Grams

G6P	Glucose-6-phosphate
gDNA	Genomic DNA
GI nematodes	Gastrointestinal nematodes
GliPR	Glioma pathogenesis-related protein
GSH	Glutathione
GST	Glutathione S-transferase
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid
HEL	Hen egg lysozyme
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HHVI	Human hookworm vaccine initiative
HPI	Hookworm platelet inhibitor
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEF	Isoelectric focusing
IFN- $\gamma$	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-4R $\alpha$	IL-4 receptor alpha
iNOS	Inducible nitric oxide synthase
IPG	Immobilised pH gradient
IPSE	IL-4 inducing principle of schistosome eggs
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
ITS-1/2	Internal transcribed spacer 1/2
J strain	Japanese strain of <i>N. brasiliensis</i>
kDa	Kilodaltons
L1/2/4	First/second/fourth stage larvae
L3	Third-stage infective larvae
L3A	Activated third-stage infective larvae
LB	Luria-Bertani
LF	Lymphatic filariasis
Lp	Lipid
LPS	Lipopolysaccharide
M	Molar
mCi	Millicuries
$\mu$ Ci	Microcuries
MgCl <sub>2</sub>	Magnesium chloride
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
Min	Minute
ml	Millilitres
$\mu$ l	Microlitres
ML	Maximum likelihood
MLN	Mesenteric lymph node
mm	Millimeters
mM	Millimolar
$\mu$ M	Micromolar
mMCP-1	Mouse mast cell protease 1
MPP <sup>type2</sup>	Multipotent progenitor type-2
mRNA	Messenger RNA
MTP	Metalloprotease
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
NaF	Sodium fluoride

NBF	Neutral buffered formalin
NBNT	Non-B-non-T cell population
NDPK	Nucleoside diphosphate kinase
ng	Nanograms
NIF	Neutrophil inhibitory factor
NJ	Neighbour-joining method
NK cell	Natural killer cell
nm	Nanometres
NME	Nucleotide metabolising enzymes
NMR	Nuclear magnetic resonance
NS	Non-significant
OD	Optical density
OVA	Ovalbumin
PAF	Platelet activating factor
PAF-AH	Platelet activating factor acetylhydrolase
PAS	Periodic acid-Schiff
PC	Phosphorylcholine
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween 20
p.i.	Post-infection
pI	Isoelectric point
PI3K	Phosphoinositide 3-kinase
PRP	Pathogenesis-related protein
Prx	Peroxiredoxin
rDNA	Ribosomal RNA gene
RELM $\alpha/\beta/\gamma$	Resistin-like molecules alpha/beta/gamma
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RMCP II	Rat mast cell protease II
RS	Rat serum
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SCID mouse	Severe combined immunodeficient mouse
SCP	Sperm-coating glycoproteins
SD rats	Sprague-Dawley rats
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEA	Schistosome soluble egg antigen
SEM	Standard error of the mean
SMC	Smooth muscle cell
STAT6	Signal transducer and activator of transcription 6
STN/STH	Soil-transmitted nematode/helminth
SSU	Small subunit gene
TAE buffer	Tris-acetate-EDTA buffer
TB	Tuberculosis
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF- $\beta$	Transforming growth factor-beta
Th1	T-helper 1
Th2	T-helper 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Tregs	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
Tris	Tris(hydroxymethyl)aminomethane
U	Units
UDP	Uridine diphosphate
UTP	Uridine triphosphate
V	Volts
VAL	Venom allergen homologue / <i>Ancylostoma</i> secreted protein-like
v/v	Volume per volume
W strain	Wellcome strain of <i>N. brasiliensis</i>
WM	Worm medium
WT	Wild-type
w/v	Weight per volume
$x g$	Centrifugal force



# **Chapter 1**

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## **General Introduction**

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## Overview

Parasitic helminths are responsible for some of the most prevalent infections which constitute a major global concern and severely afflict health conditions, particularly in the developing world. Secreted proteins are the primary interface between parasite and host, and are involved in a wide range of functions including invasion, host colonisation and regulation of the host immune response. Such strategies allow parasitic nematodes to establish long-lasting infections in the host, which account for the chronic symptoms characteristic of many helminth diseases. A better understanding of nematode secreted proteins will improve our insight into host-parasite relationships, and possibly inform on novel control strategies and therapeutic targets.

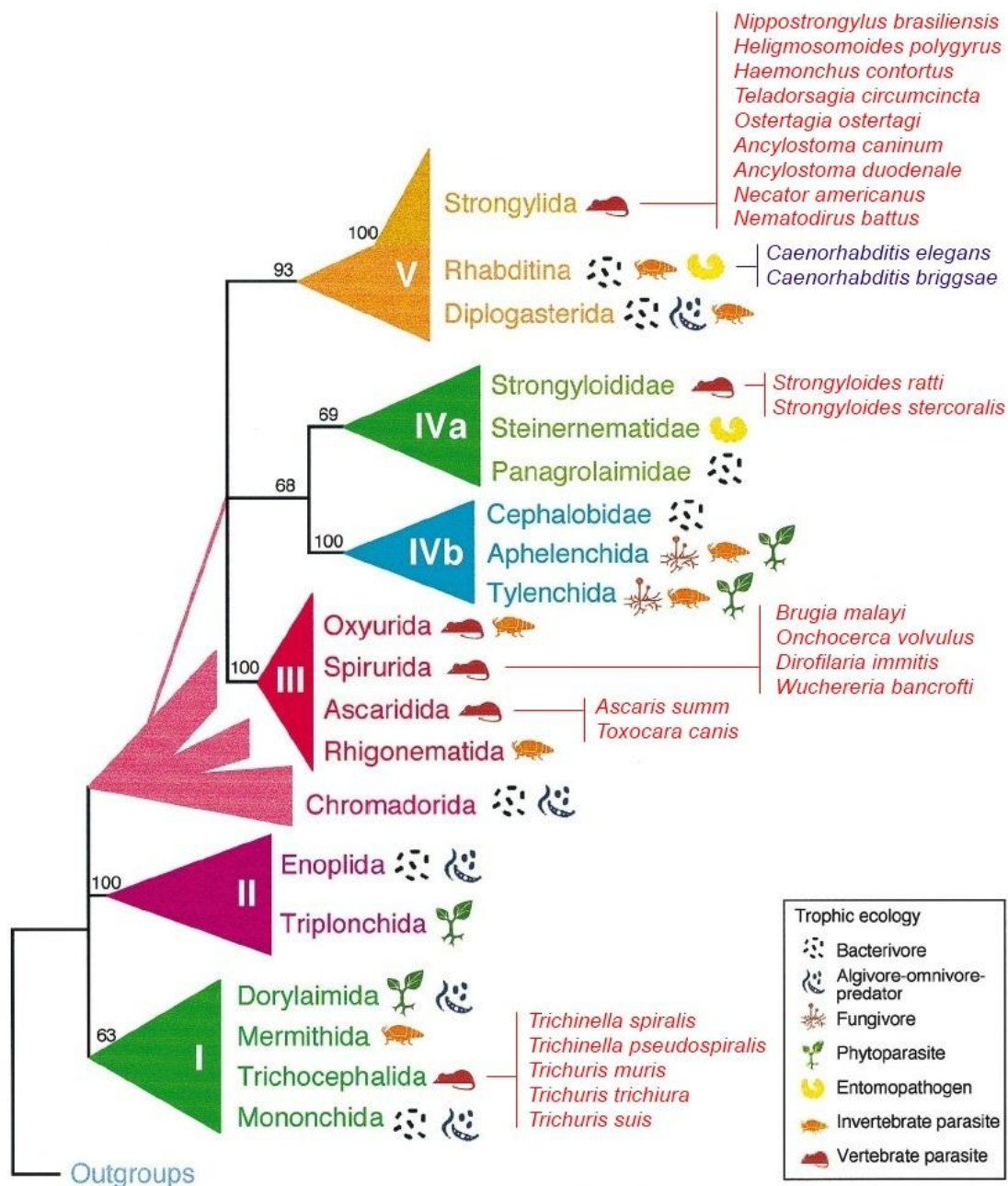
### 1.1. Helminth phylogeny

The term helminth broadly refers to four phyla of parasitic worms with superficial similarities: the *Platyhelminthes* (flatworms; includes the classes trematodes (flukes) and cestodes (tapeworms)), *Nematoda* (roundworms), *Nematomorpha* (hairworms) and *Acanthocephala* (spiny-headed worms) (Smyth, 1988). From a disease perspective, the most medically relevant parasites belong to the phyla *Platyhelminthes* and *Nematoda*.

The nematodes, or roundworms, are elongate, cylindrical worms. They are encased in a stiff cuticle, which is often extended to form a variety of structures at the anterior and posterior ends. Although the basic anatomy of nematodes is relatively uniform, the pattern of diversity is complex, with estimates of species number within the phylum ranging from 40,000 to 100 million (Lambshhead and Inst, 1993), one of the reasons why the credibility of classification based solely on morphological and ecological traits is no longer adequate. Modern classification schemes are based systematically on the molecular phylogeny of the nematodes, which, in addition to increased reliability and reproducibility, also lifts the stiff distinction between free-living and parasitic species, which gives researchers a wider scope for cross-referencing and analysis. There are several systems applied to the classification of

nematodes, but the most commonly accepted phylogenetic structure is shown in Fig. 1.1., in which the phylum *Nematoda* is divided into five clades (I-V) based on their similarities in the ribosomal RNA small subunit (SSU) sequences, and the species within each order and suborder distinguished using a combination of internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) sequence data (Audebert *et al.*, 2005; Blaxter *et al.*, 1998; Dorris *et al.*, 1999).

Under this classification system, *Nippostrongylus brasiliensis* belongs to clade V, within the order *Strongylida* and the superfamily *Trichostrongyloidea* (Chabaud *et al.*, 1965). Along with *N. brasiliensis*, many other vertebrate parasitic nematode species commonly used in research are also annotated in Fig. 1.1. The human hookworms *Ancylostoma duodenale* and *Necator americanus* lie in the same clade as *N. brasiliensis*, as well as the large ruminant cattle parasite *Ostertagia ostertagi* and the small ruminant sheep parasites *Haemonchus contortus* and *Teladorsagia circumcincta*. The model nematode *Caenorhabditis elegans*, though a free-living species, is shown in this diagram and also belongs to clade V, showing that it is quite closely related to *N. brasiliensis*. Nematodes which are parasitic to animals are loosely classified into intestinal, blood and/or tissue dwelling species, of which *N. brasiliensis* belongs to the first group.



**Figure 1.1.** The phylogenetic structure of the phylum *Nematoda*, revealed by analysis of small subunit (SSU), internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) rDNA sequences. Nematodes are divided into five clades (clades I-V), and *N. brasiliensis* belongs to clade V. Parasitic species commonly used in research are shown in red, while the free-living nematodes are shown in blue. Diagram is modified from Dorris *et al.* (1999) and annotated with additional data from Blaxter *et al.* (1998).

## 1.2. Helminth infection and disease

Parasitic helminths are responsible for some of the most prevalent diseases worldwide, accountable for debilitating chronic conditions which mostly afflict the developing world. The soil-transmitted nematodes or helminths (STN / STH) are the most common causes of helminthiases in humans. Their global prevalence far exceeds that of any other helminth class, accountable for over 2 billion cases of infection worldwide (Table 1.1). The major STHs in human disease are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), *Necator americanus*, *Ancylostoma duodenale* (hookworms), and *Strongyloides stercoralis* (threadworm). The infective eggs or larvae of these nematodes thrive in warm and moist soil (which is why such infections are most prevalent in tropical and subtropical countries), invading the host via cutaneous or oral routes. Adults can live in the gastrointestinal (GI) tract of humans for years after an infection, feeding on blood and / or host nutrients. The intensity of infection is also an important factor to take into account when considering its epidemiology, as the worm burden is often a determinant in the rate of transmission as well as the severity of symptoms in helminth diseases (Vercruysse *et al.*, 2008).

Although these infections are generally not fatal, they are often associated with high rates of morbidity. Assessments based on the disability-adjusted life year (DALY) metric, a calculation which combines the number of years lost due to ill-health, disability and premature mortality, indicate that their impact on global health is comparable to that of better-known conditions such as malaria and tuberculosis (TB) (Table 1.2) (Hotez *et al.*, 2007; Vercruysse *et al.*, 2008). Although lacking in the dramatic manifestations of filarial diseases such as elephantiasis (lymphatic filariasis; LF) and river blindness (caused by *Wuchereria bancrofti* / *Brugia malayi* and *Onchocerca volvulus* respectively), some effects of STH infection include severe anaemia, malnutrition, reduced physical development, as well as impaired memory and cognition (Crompton and Nesheim, 2002). As school-aged children are the most susceptible population group to STH infections, the extended health consequences may include stunted growth, educational performance and reduced future wage-earning capacity and worker productivity (Bleakley, 2007), in addition to a chronic state of reduced well-being. These translate into substantial poverty-promoting effects

which impede economic progress in the developing world. Another population group particularly susceptible to hookworm infection is women of reproductive age (Hotez *et al.*, 2007). The severe anaemia, iron-deficiency and overall malnutrition accompanying these infections during pregnancy result in high rates of premature or abnormal childbirth, reduced birthweight, in addition to maternal / neonatal mortality (Christian *et al.*, 2004; Dreyfuss *et al.*, 2000).

In rural areas of extreme poverty throughout the tropics and subtropics, inhabitants are often found to be chronically infected with several different species of parasitic helminths (polyparasitism) (Hotez *et al.*, 2008; Hotez *et al.*, 2007). Sufferers of such coinfections endure the additive effects (e.g. severe anaemia and malnutrition) as well as synergistic effects (e.g. increased susceptibility, intensity and transmission) of disease, resulting in conditions of extreme poor health. Such effects are not confined to helminth infections. It has become increasingly apparent that helminth infections can exacerbate disease progression and increase susceptibility to malaria and HIV/AIDS (Borkow and Bentwich, 2006; Druilhe *et al.*, 2005). Despite the serious consequences of such infections, less than 1% of global research dollars is spent on helminth research (Hotez *et al.*, 2008), thus earning such conditions the term neglected tropical diseases.

Parasitic nematodes also contribute to substantial economic losses in the livestock industry. Soil-transmitted nematodes have the largest impact on grazing animals such as cattle and sheep, causing premature mortality, malnutrition, reduced bodyweight and reduced milk production in these animals (Waller *et al.*, 1997). The major nematodes of economic significance in livestock are *Haemonchus* spp., which feed on host blood by damaging the abomasal mucosa; *Trichostrongylus* spp. and *T. circumcincta* which infect sheep; and *O. ostertagi*, which is the major disease-causing nematode in cattle. It has been estimated that control of such nematodes cost the livestock industry £1000 million every year (Newton and Munn, 1999). Additionally, inspection of swines for *Trichinella* infection, which can be passed on to humans via consumption of pork, also costs the European Union over £346 million annually (Murrell and Pozio, 2000).

Current treatment for nematode infection include broad-spectrum anthelmintics of the benzimidazole class (e.g. albendazole, mebendazole, thiabendazole, fenbendazole), which disrupt microtubule structures in helminths; as well as ivermectin and levamisole, which target invertebrate ion channels and saturate nicotinic receptors, respectively (Kohler, 2001). Although effective in the killing and expulsion of nematodes, these drugs are associated with problems of rapid reinfection and developing resistance, as well as concerns about chemical residues in the food chain (Adugna *et al.*, 2007; Conder *et al.*, 1997; Prichard, 1994). It is probable that resistance is brought about by the extensive use, repeated administration and improper dosage in the application of these drugs, resulting in the survival and propagation of nematode sub-populations which are genetically and physiologically resistant to anthelmintic treatment. These limitations highlight the need for novel control strategies, and much of the recent research has focused on the development of immunoprophylactic vaccines.

Disease	Major etiologic agent	Global prevalence	Regions of highest prevalence
<b>Soil-transmitted nematodes</b>			
Ascariasis	<i>Ascaris lumbricoides</i> (roundworm)	807 million	Developing regions of Asia, Africa, and Latin America
Trichuriasis	<i>Trichuris trichiura</i> (whipworm)	604 million	Developing regions of Asia, Africa, and Latin America
Hookworm	<i>Necator americanus</i> ; <i>Ancylostoma duodenale</i>	576 million	Developing regions of Asia, Africa, and Latin America (especially areas of rural poverty)
Strongyloidiasis	<i>Strongyloides stercoralis</i> (thread worm)	30–100 million	Developing regions of Asia, Africa, and Latin America (especially areas of rural poverty)
<b>Filarial nematodes</b>			
LF	<i>Wuchereria bancrofti</i> ; <i>Brugia malayi</i>	120 million	Developing regions of India, Southeast Asia, and sub-Saharan Africa
Onchocerciasis (river blindness)	<i>Onchocerca volvulus</i>	37 million	Sub-Saharan Africa
Loiasis	<i>Loa loa</i>	13 million	Sub-Saharan Africa
Dracunculiasis (guinea worm)	<i>Dracunculus medinensis</i>	0.01 million	Sub-Saharan Africa
<b>Platyhelminth flukes</b>			
Schistosomiasis	<i>Schistosoma haematobium</i> ; <i>Schistosoma mansoni</i> ; <i>Schistosoma japonicum</i> (blood flukes)	207 million	Sub-Saharan Africa Sub-Saharan Africa and Eastern Brazil China and Southeast Asia
Food-borne trematodiasis	<i>Clonorchis sinensis</i> (liver fluke); <i>Opisthorchis viverrini</i> (liver fluke); <i>Paragonimus spp.</i> (lung flukes); <i>Fasciolopsis buski</i> (intestinal fluke); <i>Fasciola hepatica</i> (intestinal fluke)	>40 million	Developing regions of East Asia
<b>Platyhelminth tapeworms</b>			
Cysticercosis	<i>Taenia solium</i> (pork tapeworm)	0.4 million (Latin America only)	Developing regions of Asia, Latin America, and sub-Saharan Africa

**Table 1.1. The prevalence and distribution of major helminth infection and disease.** Diagram is from Hotez *et al.* (2008).

Disease	DALYs lost annually	Deaths
Hookworm infection	22.1 million	65,000
Ascariasis	10.5 million	60,000
Trichuriasis	6.4 million	10,000
Total STH Infections	39.0 million	135,000
Tuberculosis	34.7 million	2-3 million
Malaria	46.5 million	112 million

**Table 1.2. The impact of major STHs compared to other priority diseases such as TB and malaria expressed as DALYs lost annually.** Diagram is from Vercruyssen *et al.* (2008).



### 1.3. Nematode invasion and secreted proteins

Infection generally begins with the invasion of a host. Most parasitic nematodes enter their hosts orally or by skin penetration, after which they may travel through the host's body via distinct migratory routes to arrive at their final destination / preferred niche as mature adults. Others may utilise intermediate hosts, for example filarial nematodes utilise arthropods as vectors for transmission. Embryonated eggs of *Trichuris* and *Ascaris spp.* are directly ingested, whereas larvae of the cattle and sheep nematodes *O. ostertagia*, *H. contortus* and *T. circumcincta* moult to their infective forms within faeces and soil on pastures before they are orally taken up by grazing animals (Smyth, 1988). The infective larvae of other soil-transmitted nematodes such as *N. brasiliensis*, *N. americanus*, *A. duodenale*, *A. caninum* and *S. stercoralis* invade by skin penetration, which is a less passive strategy.

Infective larvae of soil-transmitted nematodes often display negative geotropism, in that they crawl up to the highest points on objects such as grass stems, to maximise host encounter (Kassai, 1982; Smyth, 1988). They are also sensitive to host signals such as warmth, moisture and chemical signals, and they are often positively chemotactic towards such factors (Haas, 2003). This is particularly true of larvae which invade by active skin penetration, as they need to actively and accurately sense and respond to host signals, so that they can prepare and orient themselves appropriately for invasion. Animal skin is a major barrier to nematode invasion, consisting of tough layers of corneum and epidermal cells which are made to prevent entry of pathogens. The mechanics of skin penetration of nematodes has been studied in *N. brasiliensis*. Initial penetration and locomotion is facilitated by a thin film of lipid but not water, and it was observed that larvae penetrate headfirst into crevices or wrinkles (weaker interface) within the stratum corneum, where they migrate between cells down towards the epidermis and dermis within 5 minutes (Lee, 1972). The surface permeability of post-penetrative larvae appears to increase, suggesting a surface modification perhaps brought about by exsheathment of the outer cuticle (Proudfoot *et al.*, 1993). Dissolution of collagen fibres in surrounding tissue and debris were observed along the migratory tracks, suggesting that larval digestion of the connective tissue and extracellular matrix is possible, similar to enzymes secreted during skin penetration of *Schistosoma* cercaria larvae (Lee, 1972; Salter *et al.*, 2000;

Stirewalt and Fregeau, 1966). In a later study, it was found that *N. americanus* larvae also penetrated skin in a similar fashion. *N. americanus* larvae exsheath at the onset of penetration, leaving behind empty sheaths at the site of penetration, and a wake of disrupted tissue was observed along their migratory tracks (Matthews, 1982). More importantly, it was found that *N. americanus* larvae secrete an enzyme with proteolytic activity at 37°C, which may have a role in the penetrative process. Histochemical analyses of skin sections showed that migrating *Strongyloides ratti* larvae also disrupt basement membranes, dermal ground substances, collagen fibres and glycoproteins (Lee and Lewert, 1956). Moreover, live larvae and larval extracts of *S. ratti* and *A. caninum* both exhibited protease activity *in vitro* which can be abolished by treatment with heat or chelating agents, providing evidence that such enzymes are actively produced by the nematode larvae. Thus, parasitic nematodes seem to have evolved strategies to overcome the skin barrier, and several ‘invasion factors’ have been identified in their secreted proteins.

Although the mechanistic action of incoming larvae may constitute some physical force in skin penetration, secretion of hydrolytic enzymes to digest skin components may facilitate the process. Parasitic nematodes secrete a range of serine-, aspartic-, cysteine- and metalloproteases, and many of these have been implied in tissue invasion and extracellular protein digestion (Dzik, 2006). Notably, a major secretory product of *S. stercoralis* larvae was found to be a metalloprotease which can degrade a model of the dermal extracellular matrix as well as elastin and collagen substrates, which are major macrocomponents of skin and tissue (McKerrow *et al.*, 1990). Moreover, treatment with metalloprotease inhibitors blocked skin invasion by *S. stercoralis* larvae. It was also noted by the authors that elastinolytic proteases were also identified in skin-penetrating larvae of hookworms, *Onchocerca* and *Schistosoma mansoni*. The *S. stercoralis* metalloprotease was later identified to be a zinc endopeptidase and named Ss40 (Brindley *et al.*, 1995). Ss40 is immunogenic, with high titres of antigen-specific IgG found in infected humans, but however also stimulates histamine release from peripheral blood leukocytes, suggesting that it is allergenic.

Secretions of metalloproteases have also been discovered in hookworms. Both *A. duodenale* and *A. caninum* secrete enzymes with metalloprotease activities and could

degrade human fibronectin in connective tissue, but not bovine elastin or human laminin (Hotez *et al.*, 1990). This may suggest differences in their invasive properties to *S. stercoralis*, whose infection route is strictly penetrative, and could degrade all three substrates. Similar to the situation in *S. stercoralis*, metalloprotease inhibitors also inhibited larval penetration of *A. caninum* L3 into dog skin (Williamson *et al.*, 2006). A specific metalloprotease identified from *A. caninum*, Ac-MTP-1, was subsequently cloned and expressed as a recombinant protein (Zhan *et al.*, 2002). Immunobinding studies with antiserum to Ac-MTP-1 localised this protein to secretory granules and apparatus in *A. caninum* L3 (Williamson *et al.*, 2006). The antiserum also inhibited the ability of Ac-MTP-1 to digest collagen by 85% and decreased larval skin penetration by 75%, compared to only 5% and 10% reductions when treated with immune serum from multiply infected animals. Moreover, secretion of Ac-MTP-1 is induced under experimental activation upon host stimuli, suggesting that it is an important molecule at the initial stage of infection (Zhan *et al.*, 2002).

The human hookworm *N. americanus* is another obligate skin-penetrator, and a comprehensive study has been carried out to examine all classes of proteases secreted by this parasite in relation to skin invasion. Larval secretions show substantial hydrolytic activities, degrading human collagen types I, III, IV and V, as well as fibronectin, laminin and elastin (Brown *et al.*, 1999). Using a range of protease inhibitors, it was shown that all these substrates were hydrolysed by aspartyl proteases. Collagen and elastin can be hydrolysed by metalloproteases, whereas the serine proteases hydrolysed only elastin. It was further demonstrated that larval penetration through hamster skin was only inhibited by pepstatin A, suggesting that the major hydrolytic activities in *N. americanus* involved in skin penetration can be contributed to aspartyl proteases.

Apart from skin penetration, secreted proteases also have an important role for nematode invasion of other host tissues. For example, collagenolytic and elastinolytic activities are very important for *H. contortus* L4 and *Trichinella spiralis* L1 larvae in the penetration of smooth muscle and epithelial cells in the gut. Serine- and metalloprotease activities in *Onchocerca volvulus* are also highly important for the larval migratory stages through cutaneous tissues (Dzik, 2006). The invasive

properties of such parasite secretions, although equally intriguing, are however beyond the scope of this thesis and will not be further examined in this section.

Hyaluronic acid is a major component of the dermal ground substance which holds together the collagen, elastic and reticular fibres which constitute the skin layers. It also acts as a binding agent in cell-to-cell adhesion of epithelial cells and keratinocytes in skin (Miyake *et al.*, 1990). Two biologically active hyaluronidase variants have been identified in hookworm larvae of the genus *Ancylostoma* (*A. caninum*, *A. braziliense*, *A. tubaeforme*) (Hotez *et al.*, 1992) and gastrointestinal invasive stages of *A. caninum*, *Anisakis simplex* (Hotez *et al.*, 1994) and *H. contortus* (Rhoads *et al.*, 2000). The stage-specificity of hyaluronidase secretions by these parasites suggests that they may be important for tissue degradation during migration through skin and intestinal tissue. Moreover, of the hookworm species studied, *A. braziliense* showed greater hyaluronidase activity than *A. caninum* and *A. tubaeforme*, which may be relevant to the fact that it is an obligate skin-penetrating nematode which is the major cause of cutaneous larva migrans compared to the other two species (Hotez *et al.*, 1992).

Therefore, these experiments suggest that larval hydrolytic enzymes may represent major virulence factors which contribute to pathogenesis and infectivity. Strategies to target such proteins may give rise to possible therapeutic agents directed against the initial steps of infection.

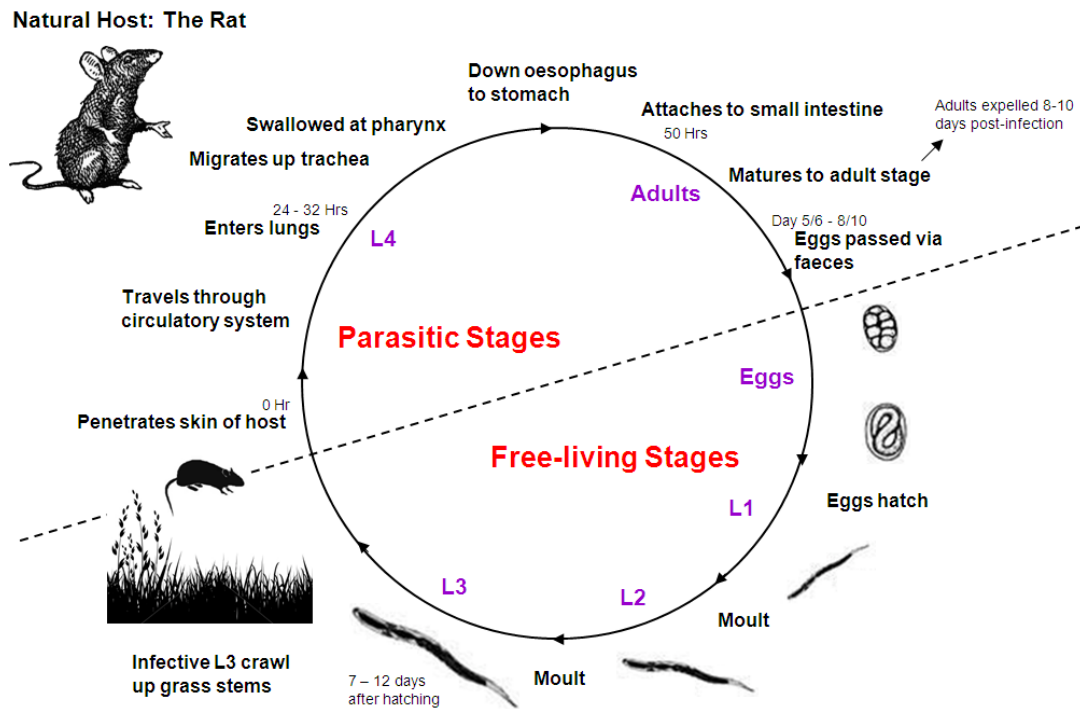
#### 1.4. *N. brasiliensis* as a model parasitic nematode

*N. brasiliensis* is extensively used as a model gastrointestinal (GI) parasite in the study of nematode infection and immunity. The widespread use of this parasite is due partly to its resemblance in life cycle and host response to other nematodes which are of medical, veterinary and agricultural importance. *N. brasiliensis* lies within the same clade as the human hookworm species *N. americanus* and *A. duodenale*, and is also very closely related to the cattle parasite *O. ostertagi* and the sheep parasite *H. contortus*, both of which cause significant agricultural and economic losses each year. Unlike some of these nematodes, *N. brasiliensis* can be easily and inexpensively maintained in the laboratory; it has a short life cycle, no intermediate hosts are required, and large quantities of worms from various stages can be obtained relatively easily. The natural and most specific host of *N. brasiliensis* is the rat, which makes laboratory manipulations more accessible and results more predictable. Infections can also be established in other rodent hosts, such as (in order of susceptibility) the mouse, hamster, ground squirrel, gerbil, rabbit and chinchilla, although the infection dynamics may vary to different degrees (Kassai, 1982). In most of these cases however, the rate of establishment is low, so gradual adaptation to the host species is required. For example, 4-7 serial passages through the mouse is needed for an enhancement in infectivity (Solomon and Haley, 1966; Wescott and Todd, 1966), and 16-30 passages are needed for adaptation to the hamster (Haley, 1966a, b). Infection with *N. brasiliensis* typically results in a highly potent and protective immune response in rodents, which are thereafter resistant against further infections (Ogilvie and Jones, 1971), therefore providing an excellent model for studying nematode immunity in both primary and secondary infections.

The life cycle of *N. brasiliensis* can be divided into an external free-living phase and a within-host parasitic phase (Fig. 1.2). The third-stage infective larvae (L3) invades its host by skin penetration, and they travel around via the host circulatory system until they pass through the lungs between 24-72 hours post-infection, where they moult into the L4 stage, migrate up the trachea, and are coughed into the host's pharynx (Ogilvie and Jones, 1971). There they are swallowed into the gastrointestinal tract and they migrate to the proximal end of the small intestine, the jejunum, where they attach to the intestinal wall as fully matured adults. The adults mate and produce eggs which

are passed out into the external environment via the host's faeces. The eggs hatch and undergo two further moults until they emerge again as infective L3, thus completing the cycle.

In the free-living stage, L3 are developmentally arrested in an environmentally-resistant, metabolically repressed phase until they encounter an appropriate host. During invasion, host signals induce L3 to develop into their parasitic phase, a point in their life history known as the 'transition to parasitism' (Hawdon *et al.*, 1992). Nematode secreted proteins are often released in a stage-specific manner. Proteins secreted during the parasitic stages can provide insight into mechanisms of host-parasite dynamics, and may have possible therapeutic value. Of particular interest are proteins secreted by L3 at its transition to parasitism, which marks the beginning of an invasion process that can be partially mimicked *in vitro* by culturing larvae at 37 °C (Bonner, 1979). As discussed in the last section, proteins secreted at this stage may contain factors which are important for invasion and the early stages of infection. The significance of this and the activation process, which is a major focus of this thesis, will be discussed in Chapter 4. Proteins secreted by the gut-dwelling adults of *N. brasiliensis* may also give important clues into host-parasite dynamics after maturation and establishment of the parasite.



**Figure 1.2: Life cycle of *N. brasiliensis*.** The different stages of *N. brasiliensis* development is shown in purple, and divided into free-living and parasitic stages. The migratory route of *N. brasiliensis* through the host is shown in black. Diagram is modified from Dunne (2001) and Ogilvie and Jones (1971).

## 1.5. Host response to infection

During experimental infections in murine hosts, the adult worm population of *N. brasiliensis* in the intestines is normally static for a number of days, and eggs are steadily produced throughout this period. At the end of this time, egg production falls rapidly as adults are expelled from the intestine under host immune mechanisms, an event generally referred to as immune expulsion (Ogilvie *et al.*, 1977). Although *N. brasiliensis* causes a relatively short-lived infection, it stimulates a powerful and protective immune response from the host. This phenomenon is common to many gastrointestinal helminths, and the biochemical basis of this event is discussed in this section.

### *The T helper 2 (Th2) response*

Infections with helminths are typically associated with a dominating T helper 2 (Th2)-mediated immune response from the host, which, in several GI nematode model systems, result in parasite clearance by expulsion from the gut (Grencis, 1997). Naive CD4<sup>+</sup> T cells are programmed to eventually differentiate into progeny that follow a Th1 or Th2 path, and the pattern of differentiation is largely determined by the cytokine environment surrounding the T cell at the time of antigen presentation (Else and Finkelman, 1998). Interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10 (Mosmann and Coffman, 1989) and IL-13 (McKenzie *et al.*, 1998a) are considered signature cytokines of the Th2 response, and are the major cytokines secreted by the host upon gastrointestinal helminth infections (Finkelman *et al.*, 1997; McKenzie *et al.*, 1998b). Th1 cells on the other hand are characterised by secretion of interferon-gamma (IFN- $\gamma$ ), tumour necrosis factor-beta (TNF- $\beta$ ) and IL-2 (Mosmann and Coffman, 1989), which induce proinflammatory responses characteristic of bacterial and viral infections. The Th1 and Th2 cells show cross-inhibitory effects through release of cytokines such as IFN- $\gamma$  and IL-10 respectively (Mosmann and Sad, 1996), which creates a balance effect in which Th1 and Th2 responses represent competing arms of the immune system (Kidd, 2003).



A wealth of evidence indicates that a Th2 response is protective to nematode infections, whereas a Th1 response is linked to susceptibility. This was demonstrated in a definitive study using *Trichuris muris*, a GI nematode of the large intestine. Mouse strains which can effectively expel *T. muris* mount a Th2-biased response during infection, whereas mice unable to resolve an infection display a Th1-dominant profile (Else *et al.*, 1992). Administration of exogenous Th2 cytokines IL-4 and IL-13 to the latter however, can restore Th2 responses and induce expulsion (Bancroft *et al.*, 2000; Else *et al.*, 1994). In human epidemiology studies, it was also found that individuals who have acquired immunity through repetitive exposure to parasitic nematodes are associated with expression of Th2 cytokines, whereas those which harbour chronic heavy infections tend to overproduce proinflammatory Th1 cytokines and develop severe intestinal inflammation (Artis and Grencis, 2008).

### ***Th2 effector mechanisms in expulsion of GI nematodes***

It is well-established that CD4<sup>+</sup> T helper cells play a major role in nematode immunity, through experimental adoptive transfers and / or *in vivo* depletion of CD4<sup>+</sup> cells in nematode infection models (Else and Finkelman, 1998). The major function of CD4<sup>+</sup> cells during nematode infections is to produce Th2 cytokines, especially IL-4 and IL-13, to activate Th2-controlled effector mechanisms and to potentiate the Th2 response.

Remarkably, adoptive transfer of CD4<sup>+</sup> T cells to severe combined immunodeficient (SCID) mice, which lack both T and B cells and would normally develop chronic infections, resulted in expulsion of *T. muris* upon challenge (Else and Grencis, 1996). Apart from demonstrating a critical role for CD4<sup>+</sup> T cells, such experiments also suggest that B cell and antibody responses are generally unimportant in primary expulsion of GI nematodes, even though infections typically result in the production of Th2-associated antibody isotypes IgG1 and IgE (Urban *et al.*, 1992). It is a puzzling feature that helminth infections induce such high amounts of both parasite-specific and non-specific IgE antibodies, and yet it does not appear to be necessary for expulsion of nematodes. It is noteworthy however, that even though expulsion in *Trichinella spiralis* is not IgE-dependent, mice deficient in IgE do experience a slight delay in expulsion as well as higher larval viability, associated with diminished mast

cell responses (Gurish *et al.*, 2004). In human populations, IgE response is generally unrelated to worm burden of *N. americanus*, but, rather, with a decreased size and weight of adult hookworms in the gut as well as lower egg production (Pritchard *et al.*, 1995). In *Strongyloides ratti*, it was found that a high level of IgG1 is correlated with decreased parasite survival, whereas IgA, which is mostly produced by mucosal cells, is negatively correlated with survival as well as fecundity (Bleay *et al.*, 2007). These experiments seem to suggest that antibodies may play a part in the weakening of parasite worms at the local site and decreasing their general fitness and well-being. In a study on *H. polygyrus*, it was found that the production of polyclonal (non-parasite-specific) antibodies can be induced by infection, which acts to limit adult fecundity, although they do not affect worm burden during a primary infection (McCoy *et al.*, 2008). However, mice lacking B cells were unable to resolve a secondary infection, unlike immunocompetent mice. This was shown to be effected by parasite-specific antibodies produced after multiple infections, particularly IgG and IgA (but not IgE), which act to reduce the number of L4s, the tissue-dwelling stage of *H. polygyrus* (Liu *et al.*, 2010; McCoy *et al.*, 2008). These antibodies responses are dependent on the presence of CD4<sup>+</sup> T cells. On the other hand, mice deficient in B cells or antibody production potential were equally competent as wild-type (WT) mice in resolving both primary and secondary infections of *N. brasiliensis* (Jacobson *et al.*, 1977; Liu *et al.*, 2010), suggesting that antibody responses are not required for immunity against this parasitic nematode.

Apart from increases in serum IgG1 and IgE, a range of cell effector mechanisms are stimulated by the Th2 response resulting from GI nematode infection, including intestinal mastocytosis, eosinophilia and goblet cell hyperplasia (Else and Finkelman, 1998; Lawrence, 2003; Urban *et al.*, 1992). Mastocytosis is promoted by Th2-type cytokines such as IL-3, IL-4, IL-9 and IL-10, and is important for the effective expulsion of several GI nematodes such as *T. spiralis* and *Strongyloides venezuelensis* (Ha *et al.*, 1983; Khan *et al.*, 1993; Knight *et al.*, 2000). However, mast cell responses are not required in expulsion of *N. brasiliensis* and *T. muris* (Betts and Else, 1999; Knight *et al.*, 2000; Mitchell *et al.*, 1983). Similarly, significant IL-5-dependent eosinophilia is often observed upon infection with GI nematodes, but was generally found to be unimportant in immune expulsion (Betts and Else, 1999; Lawrence, 2003). Goblet cell hyperplasia and mucus secretion correlate positively with resistance to

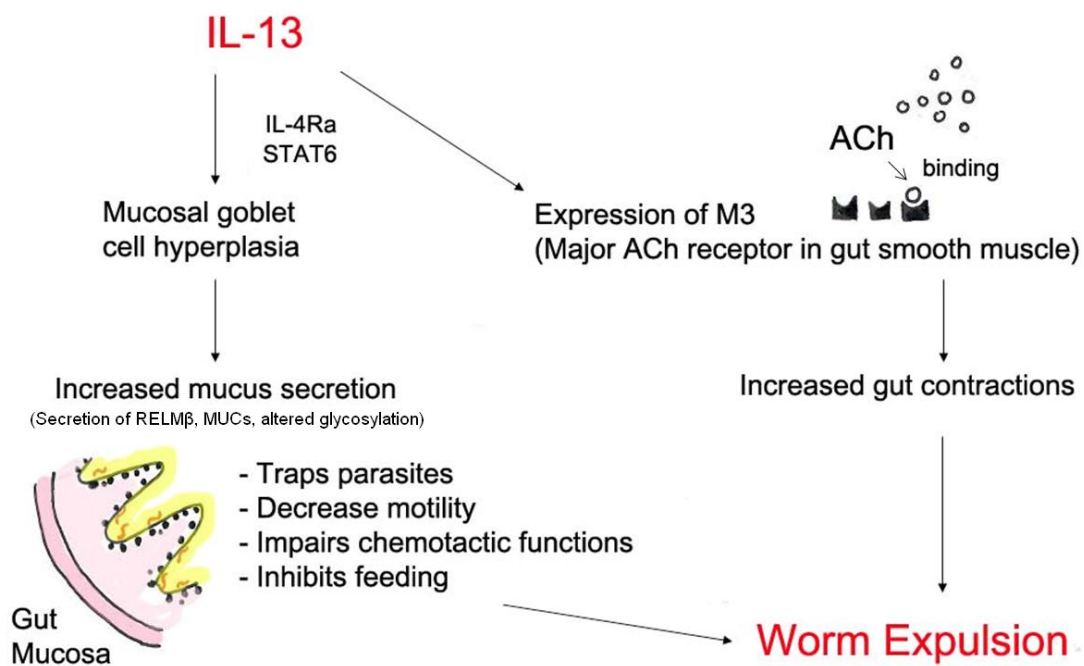
several species of GI nematodes (Else and Finkelman, 1998), and has been shown to be necessary for expulsion of *N. brasiliensis* and *T. spiralis* (Horsnell *et al.*, 2007; Khan *et al.*, 2001).

Evidently, the effective expulsion of different nematode species can require a different combination of effector mechanisms. However, a universal requirement emerges that activation of STAT6 (signal transducer and activator of transcription 6) through IL-4 and / or IL-13 signalling is essential for immune expulsion of GI nematodes including *T. muris*, *H. polygyrus*, *T. spiralis* and *N. brasiliensis* (Else and Finkelman, 1998; Lawrence, 2003). Both IL-4 and IL-13 use IL-4R $\alpha$  (IL-4 receptor alpha) as a receptor component, and can therefore elicit some overlapping functions; for example, they both have inhibitory effects on the production of proinflammatory cytokines and chemokines in monocytes (Callard *et al.*, 1996). However, it has been shown that expulsion of *N. brasiliensis* and *T. spiralis* is dependent on IL-4R $\alpha$  expression on non-bone marrow-derived cells (Urban *et al.*, 2001). Selective deletion of IL-4R $\alpha$  expression on non-bone marrow-derived cells in mice resulted in an inability to expel both nematode species, whereas deletion of IL-4R $\alpha$  expression on bone marrow-derived cells impaired normal expulsion of *T. spiralis* but not *N. brasiliensis*. Administration of exogenous IL-4 to the latter however, induced expulsion of *T. spiralis*. In general, IL-4 is more active in orchestrating and potentiating the Th2 response, as well as promoting mastocytosis and IgE production. IL-13 on the other hand has a larger role in the regulation of mucosal responses and inflammatory diseases as well as the promotion of the Th2 response (Kelly-Welch *et al.*, 2003; McKenzie *et al.*, 1998b; O'Garra, 2000). Depletion of IL-4 and/or IL-13 through the use of neutralising antibodies or knockout mice generally results in parasite persistence, and administration of these cytokines induces or enhances expulsion of a number of nematode models. Such experiments have shown that the expulsion of *T. muris* and *T. spiralis* is dependent on the presence of both cytokines (Bancroft *et al.*, 1998; Finkelman *et al.*, 2004), whereas only IL-4 is essential for protection against *H. polygyrus* (Finkelman *et al.*, 1997). In *N. brasiliensis*, IL-13 signalling was found to be critical for driving worm expulsion from the gut, while IL-4 was not required (Lawrence *et al.*, 1996; McKenzie *et al.*, 1998a; Urban *et al.*, 1998).

IL-13 signalling is transduced through IL-4R $\alpha$  and STAT-6. It has been shown that disruption of any component of this pathway blocks the host's ability to expel *N. brasiliensis* (Urban *et al.*, 1998). The IL-13/IL-4R $\alpha$ /STAT-6 signal transduction pathway is required for effective goblet cell hyperplasia, and also increases longitudinal smooth muscle cell (SMC) contraction in the intestine (Horsnell *et al.*, 2007). The principal asset of goblet cell hyperplasia is its mucus production, which is important for clearance of *N. brasiliensis* infection (Khan *et al.*, 1995; Miller *et al.*, 1981). Mucus trapping may directly affect the viability of worms through inhibition of parasite motility (Lee and Biggs, 1990), chemotactic functions (Artis *et al.*, 2004), and ability to feed (Rothwell, 1989). As for contractile responses, it was found that IL-4R $\alpha$  deficient mice suffer an inhibition of expression of the muscarinic receptor M3 (Horsnell *et al.*, 2007), which is the principal acetylcholine receptor in smooth muscle and drives 75% of contractile responses in the small intestine (Matsui *et al.*, 2002). This suggests that the IL-13/IL-4R $\alpha$ /STAT-6 pathway is involved in M3 expression and subsequently acetylcholine responsiveness, which drives SMC contractions. Together, goblet cell hyperplasia and increased SMC contractions of the gut are thought to constitute the major driving forces in immune expulsion of *N. brasiliensis* (Fig. 1.3).

Intriguingly, transcriptional levels of the resistin-like molecule (Relm) $\beta$  in mucosal cells were found to be prominently increased during infection with *N. brasiliensis* (Kawai *et al.*, 2007). Expression of Relm $\beta$  can be induced by IL-13 signalling (Artis *et al.*, 2004), and its secretion is restricted to intestinal goblet cells (Steppan *et al.*, 2001). The upregulation of Relm $\beta$  was found to coincide with the time of worm expulsion and maximal induction of protective Th2 immunity in *N. brasiliensis*, *T. spiralis* and *T. muris*, thus making it a particularly interesting target to study (Artis *et al.*, 2004; Yamauchi *et al.*, 2006). Recent data has shown that RELM $\beta$  is important for worm expulsion in *N. brasiliensis* and *H. polygyrus*. In RELM $\beta$  knockout mice, expulsion of *N. brasiliensis* was delayed, and intestinal worm numbers of *H. polygyrus* were also significantly decreased upon migration to the gut lumen (Herbert *et al.*, 2009). It was further demonstrated that while RELM $\beta$  did not affect worm viability directly, it inhibited feeding in *H. polygyrus* adults, subsequently reducing their protein and ATP content as well as fecundity. On the other hand, normal expulsion of *T. spiralis* and *T. muris* were unaffected in RELM $\beta$  knockout mice (Artis

and Grecnis, 2008; Herbert *et al.*, 2009; Nair *et al.*, 2008), which suggests that RELM $\beta$  is unimportant for immunity to nematodes which reside within the IEC layers as opposed to the intestinal lumen, where the worms are more heavily exposed to mucosal secretions. It has also been proposed that RELM $\beta$  may protect against luminal nematodes by inhibiting their chemotactic ability in location and orientation towards food source (not necessary for nematodes living within the IEC layer), thereby depleting the worms' energy sources (Herbert *et al.*, 2009). This hypothesis was extended from a study in which a link was found between RELM $\beta$  and chemotactic functions of nematode larvae. RELM $\beta$  was found to bind on the bacillary bands and cuticular pores of larval *T. muris* and *S. stercoralis* (possible locations of chemosensory receptors). Incubation of the latter with RELM $\beta$  was found to impair chemotaxis *in vitro* (Artis *et al.*, 2004), which provides some evidence for this notion.



**Figure 1.3. IL-13-dependent mucosal immunity in expulsion of *N. brasiliensis*.** IL-13 signalling results in goblet cell hyperplasia and increased smooth muscle contractions of the gut, which contribute to expulsion of *N. brasiliensis* adult worms.

As such, mucosal immunity is also extremely important for the expulsion of *T. spiralis* and *T. muris*. IL-13 signalling was found to enhance the migration and turnover of intestinal epithelial cells (IECs), which is a critical expulsion mechanism for *T. muris*, the adults of which reside within the crypts of the mucosal epithelium (Cliffe *et al.*, 2005). This forms an ‘epithelial escalator’ which propels the IECs upwards and dislodges the worms into the intestinal lumen. Dependent on STAT6 activation, goblet cell hyperplasia has been shown to be important for the expulsion of *T. spiralis* (Khan *et al.*, 2001), as are the increases in luminal fluidity and mucosal contractility which contributes to the so-called ‘weep and sweep’ mechanism in worm clearance from the gut (Madden *et al.*, 2004). Additionally, it was found that mice deficient in mouse mast cell protease 1 (mMCP-1) fail to enhance epithelial permeability during infection and experience delayed expulsion of *T. spiralis*, suggesting a role for mast cells in such responses (Knight *et al.*, 2000; McDermott *et al.*, 2003). Sensitisation of rats with *N. brasiliensis* adult antigen also resulted in a massive release of rat mast cell protease II (RMCP-II) which was shown to increase intestinal mucosal permeability (Scudamore *et al.*, 1995), although mast cell responses are not required for expulsion of this nematode (Knight *et al.*, 2000; Mitchell *et al.*, 1983). Mucin core peptide genes such as Muc2 and Muc3 show an increase in expression during infection with *T. spiralis* and *N. brasiliensis* (Kawai *et al.*, 2007; Shekels *et al.*, 2001), and expulsion of *T. muris* was found to be significantly delayed in Muc2-deficient mice (Hasnain *et al.*, 2010). The mucosal expression of a number of glycosylating enzymes are also increased during infection with *N. brasiliensis* (Kawai *et al.*, 2007), as well as alterations in post-translational glycosylation patterns of intestinal mucins not found in uninfected animals (Karlsson *et al.*, 2000; Tsubokawa *et al.*, 2009). These results suggest that the composition of mucosal secretions may have an influence on nematode immunity.

### ***Innate Type 2 responses***

Apart from playing an integral role in intestinal effector mechanisms, IECs can also act as sensors to intestinal helminthiasis. In response to infection by intestinal helminths, IECs secrete the cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which are inducers of early Th2 responses (Saenz *et al.*, 2008). TSLP was

found to directly inhibit the production of IL-12 by dendritic cells (DC), thereby influencing them towards a Th2 phenotype (Rimoldi *et al.*, 2005) which is protective to *T. muris* infection (Taylor *et al.*, 2009a). The peak expression of IL-25 and IL-33 has been found to coincide particularly with the time of establishment during infection with *N. brasiliensis* and *T. muris* respectively (Humphreys *et al.*, 2008; Hurst *et al.*, 2002). Through the use of knockout mice and cytokine administration, it was demonstrated that IL-25 and/or IL-33 are indeed protective to *T. muris* and *N. brasiliensis* infection (Fallon *et al.*, 2006; Owyang *et al.*, 2006). However, such protection seems to be dependent upon the existence of an adaptive immune response and classical Th2 mechanisms. Although IL-25 treatment confers resistance to mice normally susceptible to *T. muris* infection, severe combined immunodeficient (SCID) mice treated with IL-25 were unable to clear the infection (Owyang *et al.*, 2006). Similarly, IL-25 treatment results in accelerated expulsion of *N. brasiliensis* in mice, but only in the presence of at least one Th2 cytokine (IL-4, IL-5, IL-9 or IL-13) (Fallon *et al.*, 2006). Induced expulsion of *T. muris* in IL-33-treated mice was also associated with a protective Th2 response, including the upregulation of IL-4, IL-9 and IL-13, changes in the epithelial architecture, as well as increased goblet cell numbers and serum IgE levels (Humphreys *et al.*, 2008). Interestingly, expulsion can only be induced if the mice were treated with IL-33 early in the infection; late administration of IL-33 fails to induce protection, which suggests that these protective mechanisms act during the onset of infection. The cellular target of IL-25 and IL-33 were found to be a group of previously uncharacterised non-B-non-T (NBNT) cell population of negative lineage. These functionally similar cell types were named nuocytes, multipotent progenitor type-2 (MPP<sup>type2</sup>) and natural helper cells by independent research groups, and they promote ‘innate type 2 immunity’ to intestinal helminths through early secretion of IL-13 and/or IL-4 (Fallon *et al.*, 2006; Moro *et al.*, 2010; Neill *et al.*, 2010; Saenz *et al.*, 2010). These mechanisms are thought to be important in initiating and mediating protective immunity to intestinal helminths before the adaptive Th2 response can take hold, in a way similar to the natural killer (NK) cell response to viral infections.

Recent studies have also indicated that basophils are another innate cell population which may contribute to nematode immunity. It has been shown that systemic recruitment and expansion of basophils occur during infection with *N. brasiliensis*, *H.*

*polygyrus* and *S. venezuelensis*, the effect of which is dependent on the presence of CD4<sup>+</sup> T cells and enhanced by IL-3 (Lantz *et al.*, 2008; Min *et al.*, 2004; Mohrs *et al.*, 2005; Voehringer *et al.*, 2004). Basophils are a potent source of IL-4 (Min *et al.*, 2004; Siracusa and Artis, 2010; Sullivan and Locksley, 2009), and it was proposed that like the innate type 2 cells, they play a role in the amplification and augmentation of type 2 immunity during infection through IL-4 secretion (Sullivan *et al.*, 2011; Van Panhuys *et al.*, 2011; Voehringer, 2009). Infection with *N. brasiliensis* results in IL-4 secretion by basophils at the affected tissues during the early stages of infection, an effect which is dependent on CD4<sup>+</sup> T cells. Targeted depletion of IL-4 and IL-13 from basophils and CD4<sup>+</sup> T cells individually did not affect worm clearance during a primary infection, but combined deletion of these cytokines from both cell types significantly diminished expulsion of *N. brasiliensis*, demonstrating a contribution by basophil-derived IL-4 in primary Th2 immunity (Sullivan *et al.*, 2011). Indeed, basophils isolated from spleen, liver or bone marrow strongly biased the development of naive CD4<sup>+</sup> T cells towards a Th2 phenotype *in vitro* in an IL-4-dependent manner (Oh *et al.*, 2007). Basophils have also been shown to secrete large amounts of IL-13 *in vitro* through cross-linking of the high-affinity IgE receptor (FcεR1) on its cell surface (Falcone *et al.*, 2000), but this has yet to be confirmed in an *in vivo* model of nematode infection. Expulsion of *N. brasiliensis* in transgenic mice deficient in basophils was unimpaired during a primary infection (Ohnmacht *et al.*, 2010; Sullivan *et al.*, 2011), probably since multiple operationally redundant mechanisms are able to induce a protective Th2 response. However, Ohnmacht *et al.* (2010) found a modest delay in expulsion after secondary infection in these mice, whereas no such effect was observed by Sullivan *et al.* (2011). It is nevertheless unlikely that basophils play a large role in secondary expulsion, since the major effector responses were unaffected by basophils during reinfection, when CD4<sup>+</sup> T cells were the major source of IL-4 (Van Panhuys *et al.*, 2011).

### ***Immunity in the lungs***

Dependent on the host immune response, adult worm expulsion marks the end of an *N. brasiliensis* infection in a 'self / spontaneous cure' reaction, which means that normal, immunocompetent animals can clear the infection without extraneous help (Ogilvie



and Jones, 1971). Thereafter, hosts are strongly resistant and the parasites are cleared rapidly upon reinfection, with few or no adult worms reaching the intestines. Recently, compelling evidence showed that these worms are stopped under immune action at the lungs during a secondary infection (Harvie *et al.*, 2010). The ability of L3 to penetrate and migrate through skin was comparable in naïve and previously infected mice, but the latter showed a ten-fold reduction in the number of transiting L4 in the lungs and subsequently those reaching the gut. Tissue-specific priming with live worms in the lungs was necessary and sufficient to induce such protection, associated with an increase in CD4<sup>+</sup> T cells in mediastinal lymph nodes. Deficiency in the major histocompatibility complex (MHC) class II, STAT6 or IL-4 resulted in significantly diminished protection, indicating that a classical Th2 response is at play. The brief transition of L4 has therefore left a prolonged alteration of the immunological status of the lungs which is able to induce worm clearance during reinfection. Thus, vaccination strategies designed to directly stimulate Th2 responses in the lungs could be a possible way to interrupt reinfections and the parasite life cycle. Some support for this theory comes from vaccination studies on *S. mansoni*, in which Coulson and Wilson (1997) demonstrated that T lymphocyte recruitment to lungs is a prerequisite to prime the host for effective elimination of parasite at the lung stage.

Innate immunity to *N. brasiliensis* has also been studied at the lung stage of infection. In both SCID mice and wild-type (WT) mice, *N. brasiliensis* infection induces the expression of genes encoding molecules associated with innate immunity and tissue repair or remodelling (Reece *et al.*, 2006). However, cellular infiltrates caused by migratory larvae in the lungs were rapidly cleared in WT mice but not SCID mice. It was found that the transcription of these genes can only be sustained in WT mice, suggesting that an adaptive immune response is still needed to maintain such activities. Interestingly, one of the most prominent transcriptional changes observed in both WT and SCID mice upon infection was the upregulation of genes associated with alternatively activated macrophages (AAMΦ), *ym1*, *ym2*, *fizz1* and *arg1*. The role of AAMΦs in helminth infections is thought to include tissue healing, debris scavenging, control of inflammation, and the regulation of Th2 responses (Kreider *et al.*, 2007). Macrophages undergo alternative activation through stimulation by IL-4 and IL-13 (Martinez *et al.*, 2009), and are characterised by their secretion of YM1, arginase and

the resistin-like molecule alpha (RELM $\alpha$ , also called found in inflammatory zone 1, FIZZ1), which are likely to be of functional significance to AAM $\Phi$ s.

RELM $\alpha$  belongs to the same protein family as RELM $\beta$ , as well as RELM $\gamma$ . Unlike RELM $\beta$ , RELM $\alpha$  is primarily expressed in white adipose tissue, but is also highly expressed by AAM $\Phi$  and other cell types such as eosinophils and epithelial cells during helminth infection (Loke *et al.*, 2002; Maizels *et al.*, 2009; Steppan *et al.*, 2001). Although their amino acid sequences (49% identity) and tertiary structure are similar, RELM $\alpha$  exist as a monomer whereas RELM $\beta$  is a homodimer, as RELM $\alpha$  lacks a critical cysteine residue which is necessary for dimerisation (Banerjee and Lazar, 2001). RELM $\alpha$  has been shown to play a role in the suppression of Type 2 inflammation, a response characterised by the recruitment of Th2-related effector cells including mast cells, eosinophils, basophils, and B cells producing IgE (Nair *et al.*, 2006). Although these responses are important in mediating protective immunity, they may also provoke destructive inflammatory responses, causing collateral damage to the host. In RELM $\alpha$ -knockout mice, schistosome-induced lung inflammation was exacerbated, as well as pulmonary granuloma formation (Nair *et al.*, 2009; Pesce *et al.*, 2009). RELM $\alpha$ -knockout mice infected with *N. brasiliensis* also had intensified lung pathology, in addition to reduced fecundity and accelerated expulsion of intestinal worms, associated with an amplified Th2 response which can be dampened by treatment with exogenous RELM $\alpha$  (Pesce *et al.*, 2009). Apart from being a negative regulator of potentially destructive Th2 responses, RELM $\alpha$  has also been implied in a diverse range of functions including fibrosis at the site of infection and suppression of T cell proliferation (Horsnell and Brombacher, 2010; Nair *et al.*, 2006).

### ***Host response to migratory larvae***

Although eosinophils do not seem to be important in mediating primary expulsion of parasitic nematodes, there is evidence that they are effective in killing migratory larvae. IL-5-dependent eosinophilia (Sanderson *et al.*, 1986) has long been observed as a prominent host response upon infections with many helminth species (Urban *et al.*, 1992). However, the outcome of primary infections was not affected in studies in which IL-5 knockout hosts or anti-IL-5 antibody treatment were used in a variety of

systems such as *Trichuris muris*, *T. spiralis* and *Schistosoma mansoni* (Betts and Else, 1999; Herndon and Kayes, 1992; Sher *et al.*, 1990), thus shifting sentiment away from a role for eosinophils in helminth immunity. A turning point was the discovery that IL-5 transgenic mice with constitutive IL-5 production and lifelong eosinophilia are highly resistant to primary infections of *N. brasiliensis*, and can reduce worm burden, interfere with parasite maturation and impair fertility (Dent *et al.*, 1997; Dent *et al.*, 1999). The researchers went on to find that mice defective in IL-5 production and eosinophilopoiesis showed a lowered resistance to secondary infections, and that this response is likely to target worms at the larval stage before they reach the lungs (Knott *et al.*, 2007). Similarly, IL-5 knockout mice also experience delayed expulsion and greater worm burden of *T. spiralis* during a challenge infection, associated with decreased eosinophilia in host tissue (Vallance *et al.*, 2000). Additionally, depletion of IL-5 by treatment with neutralising antibodies in mice resulted in a greater recovery of *S. venezuelensis* worms at the lung stage after a secondary infection (Korenaga *et al.*, 1991). Evidence for the direct killing of tissue migratory larvae comes from *in vivo* experiments in which diffusion chambers containing *S. stercoralis* larvae were implanted into subcutaneous tissue of mice. Eosinophils were the only cell type that accumulated in the diffusion chamber coincident with the killing of larvae (Rotman *et al.*, 1996). Moreover, inhibition of eosinophil migration into the chamber or treatment with anti-IL-5 prevented such killing (Abraham *et al.*, 1995; Herbert *et al.*, 2000).

It was further proposed that the complement system plays a role in eosinophil-mediated responses. Complement and C3 deposition on the parasite surface facilitate the recruitment and attachment of cytotoxic effector leukocytes, including eosinophils (Giacomin *et al.*, 2005), which release products that can damage, immobilise and/or kill the parasite (Butterworth, 1984; David *et al.*, 1980). Indeed, eosinophil recruitment was reduced in mice deficient in factor B, a molecule involved in the alternative pathway of complement activation (Giacomin *et al.*, 2008). However, eosinophil action was not sufficient to eliminate the parasite, because C3 and leukocytes can only adhere specifically to worms at the L3 larval stage of *N. brasiliensis* (Giacomin *et al.*, 2005). L3 worms can moult into the L4 stage within 24 hours of entering the host, which can no longer be targeted by complement. These data suggest that L3 of *N. brasiliensis* may be vulnerable to eosinophil-based cytotoxic attacks with possible input from the complement system.

### *The 'modified Th2' environment*

Although a diverse array of type 2 responses is stimulated by helminth infections, it is apparent that immunity to different species requires a different range of protective mechanisms. Moreover, the effector mechanisms generated towards different life cycle stages in different host organs may also operate in a context-dependent manner. The mechanisms which lead to the universal induction of the type 2 immune response by almost every helminth species are not well understood. It is unlikely that each species has individually evolved its own strategy to induce the same type of response, so it is probable that underlying factors exist. One hypothesis is that the innate immune system recognises conserved helminth-associated molecular patterns as 'danger signals', preferentially generating a type 2 response, in a way comparable to the Toll-like receptor (TLR) pattern recognition mechanism which triggers type 1 responses (Kapsenberg, 2003; Van der Kleij *et al.*, 2002). A more popular theory suggests that helminths may have evolved to take an active part in the induction of the type 2 response (Maizels *et al.*, 2004). As Th1 and Th2 responses represent competing arms of the immune system, polarisation towards Th2 will result in a downplay of Th1-induced inflammation, which minimises damage to both parties (Allen, 1997). Considering the dynamic host-parasite relationships which determine the outcome of selection, the generation of type 2 immunity is an evolutionarily appropriate response to worms.

While the type 2 response is shown to be protective against helminths, this does not explain the chronic nature of some infections such as hookworms. It is a feasible thought that type 2 mechanisms working at their full power may have the potential to clear these normally chronic infections, but at the cost of generating excessive immune damage to the host in the process. It may thus be the lesser of two evils for the host to tolerate and accommodate such infections; and helminths, between a fine balance of host mortality and its own survival and propagation, may have evolved to dampen rather than disable the immune response directed against them, resulting in a 'modified Th2' environment (Allen and Maizels, 2011; Maizels *et al.*, 2004). The concept of a pathogen as an active participant involved in the generation of the host immune response is a process known as immunoregulation.

## 1.6. The role of secreted proteins in immunoregulation

Immunoregulation is a universal concept defined by the suppression, diversion or conversion of the host immune response by a pathogen, usually to its advantage. Such strategies allow parasitic helminths to establish long-lasting relationships in the host by escaping immediate elimination, which accounts for the chronic features seen in many helminth infections. The generation of a favourable anti-inflammatory environment through the induction of a 'modified type 2' response is a hallmark of helminth infection, and increasing evidence has shown that parasite secreted proteins can play a central role in this phenomenon.

It has been shown that *N. brasiliensis* secreted proteins can actively skew the immune response towards a Th2 pathway. Mice immunised with *N. brasiliensis* adult secreted products demonstrate the Th2 hallmarks of an infection without the need of any exposure to live parasites, an effect which can be abolished by heat or protease treatment of the parasite preparations, suggesting that these effects are likely to be caused by proteins (Holland *et al.*, 2000). This phenomenon is unaffected by the co-administration of complete Freund's adjuvant (CFA), a pro-Th1 cocktail, indicating that the observed bias towards a Th2 response is not a 'default' in the absence of Th1 stimuli. Moreover, the Th2 bias driven by *N. brasiliensis* secreted products can also be extended to third-party bystander antigens such as hen egg lysozyme (HEL), indicating that *N. brasiliensis* secreted products act as general Th2 adjuvants. Importantly, exposure to these secreted products can influence the maturation of bone marrow derived dendritic cells (BMDC) towards a Th2 phenotype which secretes IL-6 (which inhibits Th1 differentiation; Diehl and Rincon, 2002) and downregulates IL-12, and adoptive transfer of these treated cells to naive mice elicited potent Th2 responses (Balic *et al.*, 2004). As dendritic cells (DC) are important antigen-presenting cells (APC) required for the priming and activation of CD4<sup>+</sup> T lymphocytes whose path of differentiation is critically dependent on its cytokine environment at the time of antigen presentation (Else and Finkelman, 1998), this could subsequently direct T cell function towards a Th2 phenotype. Other parasite-secreted products which has been shown to prime DCs for such Th2 responsiveness include ES-62 of the filarial nematode *Acanthocheilonema viteae* (Whelan *et al.*, 2000), soluble egg antigen (SEA) and larval secretions of *S. mansoni* (Jenkins and

Mountford, 2005; MacDonald *et al.*, 2001), and the schistosome-associated glycan lacto-N-fucopentatose III (Thomas *et al.*, 2003). Additionally, *N. brasiliensis* adult secreted products was found to potently suppress mitogen-induced production of IFN- $\gamma$  and IL-2 in mesenteric lymph node cells, and also inhibited secretion of IFN- $\gamma$  from purified lymphocytes (Uchikawa *et al.*, 2000), suggesting that they may have direct modulatory effects on T cell functions as well.

Likewise, exposure to secreted products from *Toxocara canis* biased human peripheral blood lymphocytes to a Th2 phenotype with increased secretion of IL-4 and IL-5, and reduced IFN- $\gamma$  and IL-2 (Del Prete *et al.*, 1991). Schistosome SEA is known to possess potent Th2-polarising properties, and individual molecules have been attributed to this phenomenon. Alpha-1 (also called IPSE; IL-4 inducing principle of schistosome eggs) induces IL-4 secretion by basophils in an antigen-independent manner, and it also binds and sequesters chemokines to inhibit recruitment of inflammatory cells (Schramm *et al.*, 2003; Schramm *et al.*, 2007). Indeed, the depletion of Alpha-1 resulted in increased egg-induced inflammation during infection with *S. mansoni* (Smith *et al.*, 2005). Omega-1, a ribonuclease found in schistosome SEA, conditions DCs *in vitro* to drive Th2 responses, and injection of omega-1 in mice elicited a Th2 response *in vivo* (Everts *et al.*, 2009).

Secretory products of *N. brasiliensis* larvae have also been shown to downmodulate Th1-associated inflammation *in vivo*. Administration of *N. brasiliensis* L3 secreted products to rats reduced neutrophil recruitment to the lungs (Keir *et al.*, 2004) as well as the transcription of proinflammatory molecules such as IL-1 $\beta$ , tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and inducible nitric oxide synthase (iNOS) against a background of LPS-induced inflammation *in vivo* (Zhao *et al.*, 2009). Similar findings were obtained during *in vitro* stimulation of alveolar macrophages with L3 secreted products, in which both TNF- $\alpha$  and iNOS levels were again suppressed (Zhao, 2009). Whether this effect was caused by a suppression of classically activated macrophages (CAM $\Phi$ ) of the Th1 phenotype or the induction of AAM $\Phi$ s however, remains to be addressed in this model. AAM $\Phi$ s are important cells activated during helminth infection which can promote Th2 responses and suppress Th1 inflammation (Herbert *et al.*, 2004; Loke *et al.*, 2000), and there has been evidence that helminth secretory products can directly induce their activation. It was demonstrated that a peroxiredoxin (Prx)

secreted by the blood and liver flukes (*S. mansoni* and *Fasciola hepatica* respectively) can induce the activation of AAMΦs in an IL-4/13-independent manner (Donnelly *et al.*, 2005; Donnelly *et al.*, 2008). Prx stimulated the expression of markers of AAMΦs such as Ym1 in macrophages, which induced the secretion of IL-4, IL-5 and IL-14 in naive CD4<sup>+</sup> T cells. Immunisation of mice with Prx or treatment with anti-Prx antibodies resulted in a blockage of AAMΦs induction and diminished Th2 responses *in vivo* during a challenge infection with *F. hepatica*. Therefore, the induction of AAMΦs can be one of the helminth immunomodulatory strategies in tipping the balance towards Th2. Collectively, these results demonstrate the suppressive effects of helminth secreted products on proinflammatory Th1 responses.

Extending on this, much evidence has supported an inverse relationship between helminth infections and inflammatory disorders mediated by Th1 and / or the recently described Th17 response (characterised by the production of IL-17, IL-6 and TNF-α), such as rheumatoid arthritis, inflammatory bowel diseases (IBD), type 1 diabetes and multiple sclerosis (Elliott *et al.*, 2007; Zacccone *et al.*, 2006). Experimental infections with various helminth species have also been proven on several counts to be protective to such diseases. In particular, the use of *Trichuris suis* and *S. mansoni* as therapeutic agents for IBDs and multiple sclerosis are being clinically considered and has shown promising results (Croese *et al.*, 2006; Reddy, 2010; Summers *et al.*, 2005a; Summers *et al.*, 2005b). Current research has indicated that secretory products from helminths may act as immunomodulators to mediate this phenomenon. Treatment with *A. caninum* and *S. mansoni* secretory products were found to reduce inflammatory symptoms and the production of proinflammatory cytokines IFN-γ and IL-17 in a model of ulcerative colitis in mice (Ruysers *et al.*, 2009). Exposure to ES-62, a secreted protein discovered in *A. viteae* and present in many other filarial species including *B. malayi*, *B. pahangi* and *O. volvulus*, prevented the initiation of collagen-induced arthritis in murine models, and also suppressed the progression of established disease (Harnett *et al.*, 2004; McInnes *et al.*, 2003). These effects were associated with a decrease in the production of proinflammatory cytokines, demonstrating the immunomodulatory effects of ES-62. Many of the anti-inflammatory effects of ES-62 appeared to be dependent on post-translational substitutions which conjugate phosphorylcholine (PC), a hapten-like moiety, to N-linked glycans on the protein backbone, as the PC moiety chemically attached to an irrelevant molecule was

capable of mimicking the effects of ES-62 (Harnett and Harnett, 1993; Houston and Harnett, 1999). In one mode of action, PC residues on ES-62 were found to be responsible for interactions with TLR4, which in mast cells inhibits the release of inflammatory mediators (Goodridge *et al.*, 2005). PC and related modifications are also present in other secreted products of many filarial nematodes, such as an *N*-acetylglucosaminyltransferase of *B. malayi* and Juv-p120, a major secreted protein of *Litomosoides sigmodontis* (Hewitson *et al.*, 2009; Houston and Harnett, 1999).

Intriguingly, a number of studies drawing from epidemiological and experimental data have also indicated that helminth infections can protect against the pathologies of allergic inflammation, which are themselves Th2-mediated (Maizels, 2005; Wilson and Maizels, 2004). Such diseases, including asthma, hayfever, allergic rhinitis and atopic dermatitis, are characterised by the production of IgE and Th2 responses. In the case of asthma, eosinophil infiltration into the airway epithelium, the release of proinflammatory mediators and secretory mucins from goblet cells account for typical inflammatory symptoms which can cause substantial tissue damage. Notably, live infection or sensitisation with larval secretory products of *N. brasiliensis* in mice was shown to reduce inflammation in the lungs and airways respectively in a model of ovalbumin-induced asthma (Trujillo-Vargas *et al.*, 2007; Wohlleben *et al.*, 2004). While it is established that the type 2-biased response elicited by helminths provides a mechanism to counteract type 1 inflammation, this does not explain how helminths suppress type 2 allergic disorders. Recent research has shown that this phenomenon is likely to be mediated by regulatory strategies which can dampen type 2 responses through IL-10, transforming growth factor-beta (TGF- $\beta$ ) and a population of T cells with an immunosuppressive phenotype called regulatory T cells (Tregs).

While IL-10 and TGF- $\beta$  are immunosuppressive cytokines in their own rights, they were found to potentiate their effect by inducing the expansion of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3-expressing T regs, which interfere with T cell activation and secrete IL-10 and TGF- $\beta$  (Belkaid, 2007). These responses are commonly induced during helminthiasis. Infection with *H. polygyrus*, as well as the filarial nematodes *B. malayi* and *L. sigmodontis*, induced the *in vivo* expansion of Tregs which show immunosuppressive activities *in vitro* (Finney *et al.*, 2007; McSorley *et al.*, 2008; Rausch *et al.*, 2008; Taylor *et al.*, 2009b), suggesting that they may be favourable to parasite persistence.



Indeed, depletion of Tregs impaired the survival and fecundity of *L. sigmodontis* in the host, associated with an amplified Th2 response (Taylor *et al.*, 2009b). The further observation that inoculation of dead worms failed to elicit Treg responses suggest that its induction is a process actively regulated by the parasite (McSorley *et al.*, 2008). Indeed, it was found that DCs exposed to *H. polygyrus* secreted products induced the differentiation of Tregs which produced IL-10 and inhibited T cell proliferation (Segura *et al.*, 2007). *S. mansoni* has been found to activate Tregs by stimulating host TGF- $\beta$  production through secretion of SEA (Zaccone *et al.*, 2009), but *H. polygyrus* and *Teladorsagia circumcincta* can also induce Tregs directly by secreting a TGF- $\beta$  mimic which could bind to the host TGF- $\beta$  receptor, once again demonstrating the active immunomodulatory effects of nematode secreted proteins (Grainger *et al.*, 2010). Moreover, a TGF- $\beta$  homologue has also been found in *B. malayi* (TGH-2), and was shown to bind to the mammalian receptor (Gomez-Escobar *et al.*, 2000). It thus appears that the utilisation of secretory products to exploit Treg function could be a quite a common helminth strategy to suppress host immunity.

As discussed, TGF- $\beta$  is a helminth-secreted mammalian cytokine mimic with immunosuppressive functions. Another form of cytokine mimicry is demonstrated by the macrophage migration inhibitory factor (MIF) secreted by *B. malayi* and *Ancylostoma ceylanicum*, with homologues also present in a variety of other nematodes (Vermeire *et al.*, 2008). The mammalian MIF is a protein which potentiates proinflammatory gene expression and mediates directed chemotaxis and recruitment of immune cells. It is quite surprising that earlier studies indicated that the nematode-derived MIFs appeared to induce similar effects as the mammalian MIFs by inducing directed chemotaxis of monocytes / macrophages (Pastrana *et al.*, 1998) and the production of proinflammatory cytokines (Cho *et al.*, 2007; Zang *et al.*, 2002). However, it was later demonstrated that the *B. malayi* MIF synergises with IL-4 to induce the development of AAM $\Phi$ s with a potent immunosuppressive phenotype (Prieto-Lafuente *et al.*, 2009). Moreover, MIF also induced macrophages to upregulate the expression of IL-4R $\alpha$ , thereby potentiating IL-4/13 responses in a Th2 environment, producing an anti-inflammatory effect.

Helminth secreted proteins have also been shown to downplay inflammation by interfering with leukocyte recruitment. A protein named *SmCKBP*, identified from *S.*

*mansoni* SEA, was able to bind to the host chemokine CXCL8, thereby inhibiting its interaction with the host chemokine receptor (Smith *et al.*, 2005). This protein blocked CXCL8-induced migration and infiltration of neutrophils in a mouse air pouch model and in a chemotaxis model, and additionally suppressed inflammatory responses *in vivo*. The *Ancylostoma*-secreted neutrophil inhibitory factor (NIF) was found to bind as an antagonistic ligand to the CD11b subunit of the chemokine receptor CR3 (complement receptor 3), which blocks leukocyte adhesion to vascular endothelial cells and hydrogen peroxide release by activated human neutrophils (Moyle *et al.*, 1994; Rieu *et al.*, 1995). In another study, microfilarial secreted products from *B. malayi* showed potent inactivation of chemotaxis in human granulocytes, an effect brought about by cleavage of the complement anaphylatoxin C5a by a serine protease secreted by the parasite (Rees-Roberts *et al.*, 2010). Secretory products of *T. spiralis* infective larvae also inhibited C5a-mediated chemotaxis of human granulocytes, but this was found to be effected through carboxypeptidase activity (Rees-Roberts *et al.*, 2010).

It is known that helminth secretions are generally rich in protease and protease inhibitors, and some of their functions have been implied in immunoregulation. Several proteases secreted by *S. mansoni*, *F. hepatica* and *Dirofilaria immitis* were shown to degrade host immunoglobulins as a possible way of evading host immune responses (Dzik, 2006; McKerrow *et al.*, 2006). Recently, it was discovered that the major cathepsin cysteine proteases secreted by *S. mansoni* and *F. hepatica* were able to inhibit nitric oxide production, IL-12, IL-6 and TNF production by macrophages through endosomal degradation of TLR3, thus inhibiting subsequent signalling (Donnelly *et al.*, 2010). This inhibited Th1 responses and protected mice from LPS-induced lethality. On the other hand, helminth-secreted protease inhibitors may have a role in the degradation or protection against host proteases (McKerrow *et al.*, 2006). A number of cystatins (cysteine protease inhibitors) secreted by filarial nematodes were found to suppress T cell proliferation, whereas some of the serpins (serine protease inhibitors) secreted by schistosomes appeared to have anti-coagulation properties (McKerrow *et al.*, 2006). Cystatins secreted by *N. brasiliensis*, *A. viteae*, *B. malayi* and *O. volvulus* have also been shown to have immunomodulatory effects, through the inhibition of host cysteine proteases required for antigen-processing, or by induction of immunosuppressive cytokines such as IL-10 (Hartmann and Lucius,

2003; Hewitson *et al.*, 2009). Treatment with a filarial cystatin has additionally been shown to suppress Th2-related allergic inflammation as well as Th1-mediated colitis in mice, an effect mediated by Tregs and IL-10-producing macrophages (Schnoeller *et al.*, 2008).

Most helminth parasites also secrete a range of antioxidants, including superoxide dismutases, catalases, glutathione peroxidises, thioredoxin peroxidises, and peroxiredoxins, which are thought to have a role in protecting the parasites against reactive oxygen species generated by the host (Hewitson *et al.*, 2009). Interestingly, a distinctive pathway involving the exploitation of host nucleotide signalling has been proposed for *T. spiralis* in the evasion of host immune mechanisms. Tissue damage typically results in a massive release of extracellular nucleotides, which are utilised by the host immune system as ‘danger signals’, transduced through purinergic receptors (hence the term purinergic signalling). Signalling through the purinergic receptor P2 by ATP, ADP, UTP and UDP can activate or reinforce a broad range of inflammatory responses, including platelet aggregation, release of proinflammatory mediators from granulocytes and recruitment of inflammatory cells (Gounaris and Selkirk, 2005). Such signalling also stimulates the release of chloride from epithelial cells which drives net fluid influx into the intestinal tract, and increases mucus secretion from goblet cells, resulting in an unfavourable environment for intestinal helminths (Bucheimer and Linden, 2004; Leipziger, 2003). To circumvent this situation, the gut-dwelling stage of *T. spiralis* was found to secrete a set of ‘nucleotide metabolising enzymes’ to degrade or convert extracellular nucleotides. A secreted nucleotide diphosphate kinase (NDPK) was first discovered, which can convert a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP) (Gounaris *et al.*, 2001). A 5'-nucleotidase (5-NT) hydrolyses ADP and UDP to their respective nucleoside monophosphate, then AMP into adenosine and an inorganic phosphate (Gounaris, 2002; Gounaris *et al.*, 2004). This mechanism proposes a way in which *T. spiralis* is able to decrease the availability and concentration of extracellular nucleotides, thus blocking the subsequent attack by the host immune response. Interestingly, this mechanism shows striking parallels to a strategy utilised by haematophagous arthropods, which secrete enzymes in their saliva that degrade nucleotides so to minimise the ensuing pain and inflammatory responses (Gounaris and Selkirk, 2005; Ribeiro and Francischetti, 2003).

The ability of helminths to regulate the host immune response is key to their success in prolonging survival in the host and optimising its own propagation. From the existing data, it is clear that helminths may actively manipulate the host response to their advantage, a phenomenon in which secreted proteins play a large role. The strategies employed are diverse: from the induction of Th2 differentiation, the suppression of Th1 responses, the manipulation of Treg cells and AAMΦs, to the inhibition of immune cell recruitment and host signalling. The extent and combination of these strategies utilised by different helminth species may differ, as may their different lifestyles, niches and life cycle stages. However, the ultimate goal in such immunoregulation is quite clear: to produce an anti-inflammatory environment which is favourable to parasite survival and propagation, via the generation of a 'modified' Th2 response.

## 1.7. Vaccination strategies and nematode secreted antigens

Vaccine candidates for helminth infections can be categorised into three main types: worm surface antigens, hidden antigens, and secreted antigens (Munn, 1997). Worm surface antigens are quite literally molecules and epitopes found on the surface of the worm body. While it seems quite logical to assume that targeting these antigens is likely to be the most effective by weakening, killing and ultimately eliminating the worms directly, in reality this is not always the case. Most nematode species have very tough outer cuticles, which can often withstand immune damage. Moreover, nematodes undergo multiple moults during their parasitic stages in the host, resulting in a change in surface epitopes every time they shed a new layer (Philipp *et al.*, 1980; Proudfoot *et al.*, 1993), making them hard to target. Hidden antigens are not normally exposed to the immune system during a natural infection (such as those in the interior part of the worm soma), so they are less likely to have experienced selective pressure in parasite evolution to evade the immune response (Munn, 1997), i.e. a lower risk of antigenic variation. Although there has been success in the use of the nematode gut membrane antigens such as H11 and O12 for vaccination against the GI nematodes of livestock ruminants (*H. contortus* and *Ostertagia spp.* respectively) (Munn, 1997; Newton and Munn, 1999), it is not always easily determined whether protective immunity can be generated against other inaccessible antigens. It can also be argued that the majority of hidden antigens within the parasite soma are more likely to be released by damaged or dead worms, so that immune responses against such proteins would be futile. General somatic extracts are often not protective towards parasitic nematodes, so research attention has focused on parasite secretions instead (Maizels *et al.*, 1999).

Secreted proteins are essential to parasite survival. Early studies have shown that vaccination with attenuated live, but not dead, parasites can confer nematode resistance, suggesting that protective antigens are actively secreted by the parasites (Knox, 2000). Vaccination with x-ray attenuated larvae of *A. caninum* induced up to 90% protection in dogs, although this vaccine was later commercially discontinued due to its high production costs, short shelf life and respiratory side-effects (Miller, 1971, 1978). Vaccination with attenuated worms was found to be protective against many other nematode species, and commercially successful vaccines for the

lungworm in cattle (*Dictyocaulus viviparus*) and sheep (*D. filaria*) were produced using this approach (Jarrett *et al.*, 1958; Sharma *et al.*, 1988). Vaccination of humans with attenuated nematode larvae however, is generally deemed unacceptable for obvious safety and ethical reasons.

Some secreted proteins are highly immunogenic, as they are recognised by the host during natural infections, so vaccination with such proteins could possibly augment host immunity during natural re-infections. However, whether these proteins will have functional significance in protecting the host can only be determined by vaccination experiments. Early experimentation studied the protective effects of whole or fractionated parasite secreted proteins. Immunisation with *N. brasiliensis* larval or adult secreted proteins both resulted in greatly reduced intestinal worm burden in rats or mice during challenge infections (Day, 1979; Rhalem *et al.*, 1988; Thorson, 1953). Partial protection can also be achieved by immunising dogs with oesophageal extracts from *A. caninum* (Thorson, 1956). Interesting, proteolytic and lipolytic activities in these preparations are inhibited by antiserum from immune animals, suggesting that such secreted enzymes could be important targets (Thorson, 1954, 1956). Vaccination with adult secreted proteins of *Trichostrongylus colubriformis*, and larval secreted proteins of *H. contortus* and *T. circumcincta* have been found to induce variable protection from 30-70% in sheep to homologous challenge, in which different fractions generally show differential extents of protection (Emery, 1996; Newton and Munn, 1999). Immunisation with *T. spiralis* secreted proteins from muscle-stage larvae also provided protection to pigs and mice (Campbell, 1955; Murrell and Despommier, 1984).

However, vaccination with whole secreted proteins may not always be an effective strategy against all nematode species; for instance immunisation with adult secreted proteins did not confer protection to *H. polygyrus* (Day, 1979). Although whole secreted protein preparations contain a complex mix of molecules, not all of them will be protective, and some may even be immunomodulatory molecules which can act to suppress or divert the protective immune response (Meeusen, 1996). Moreover, it is probable that at least some components of secreted proteins contain allergenic epitopes – indeed, repeated exposure to larval secreted proteins of *N. brasiliensis* was shown to induce a parasite-specific airway inflammatory response with acute

infiltration of inflammatory cells in the airway and lungs (Marsland *et al.*, 2005), which is a particularly dangerous characteristic in a vaccine candidate because repeated exposure to these antigens will be needed for the generation of acquired protective immunity. Collection of nematode secretory products in sufficient amounts to produce commercial vaccines by *ex vivo* cultivation is also impractical, considering the minute amount of proteins parasitic nematodes secrete. It was estimated by Emery *et al.* (1993) that the sacrifice of three donor sheep is needed to generate sufficient parasite materials to vaccinate one sheep, making this an unviable option.

Therefore, research efforts are better spent on the study of selected molecules rather than crude parasite mixtures, so that targets can be produced industrially by recombinant protein engineering. With the advance of various technologies in protein analysis and manipulation, there has been some success in the discovery of individual protective targets. Immunisation with acetylcholinesterase (AChE), a major parasitic nematode secreted enzyme considered important for parasite survival, was found to reduce *N. brasiliensis* egg output (Ball *et al.*, 2007). A number of other nematode species including *H. contortus*, *H. polygyrus*, *T. circumcincta* and *T. colubriformis* also secrete AChE, and it was previously found that immune animals produce antibodies to this enzyme (Ogilvie *et al.*, 1973). Immunisation with AChE purified from *T. colubriformis* resulted in cross-protection against a mixed infection of *H. contortus*, *C. oncophora* and *T. colubriformis* in sheep, but with inconsistent results (Griffiths and Pritchard, 1994). Vaccination with whole unfractionated secreted protein preparations of *D. viviparus* did not elicit protection, but worm burden was significantly reduced in animals which received a fraction particularly enriched with AChE (McKeand *et al.*, 1995).

Many proteases are abundantly secreted by nematodes, and they are thought to be important in various biological functions including immunomodulation, host invasion and nutrient acquisition. The secretion of digestive proteases is assumed to be important for blood-feeding nematodes for the extracellular degradation of host haemoglobin and other serum proteins, so to derive peptides and amino acids which can be transported across their intestinal lumen. Digestion of haemoglobin is initiated under the synergistic action of the aspartic protease APR-1 and the cysteine protease CP-2, and the metalloprotease MEP-2 was able to cleave the resulting globin

fragments (Williamson *et al.*, 2004). Based on this rationale, significant research progress has been made in hookworm vaccines by targeting their digestive proteases as a strategy to cut off their nutrient supply and subsequently survival and fecundity (Pearson *et al.*, 2010). Vaccination with *A. caninum* AcAPR-1 in dogs resulted in a 33% reduction in intestinal worm burden and a 70% reduction in faecal egg counts upon homologous challenge, and additionally reduced blood lost and anaemia in the host (Loukas *et al.*, 2005). IgG from the vaccinated dogs inhibited APR-1 activity *in vitro*, and also bound *in situ* to the worm intestines, suggesting that the neutralising antibodies are ingested by the worms during feeding which can effectively inhibit their ability to digest blood. Hamsters immunised with AcAPR-1 also displayed a 44% reduction in worm burden upon heterologous challenge with *N. americanus*, indicating that cross-protection can be extended to human hookworms (Xiao *et al.*, 2008). So promising were the protective effects of this antigen that the *N. americanus* Na-APR-1 is now the leader candidate under trials for the Human Hookworm Vaccine Initiative (HHVI, Sabin Institute) (Periago, 2010).

Immunisation with the *A. caninum* Ac-CP-2 cysteine protease also resulted in partial protection in dogs, resulting in decreased fecundity and stunted growth, however with no significant decrease in intestinal worm burden, even though the mode of vaccine action was similar to APR-1 (Loukas *et al.*, 2004). Hamsters vaccinated with Na-CP-2 on the other hand, showed a small but significant reduction in worm burden by 29.3% (Xiao *et al.*, 2008). In *H. contortus*, it has been estimated that 16% of the adult transcriptome encode for cysteine proteases (Jasmer *et al.*, 2004), and many of these are associated with activities in anticoagulation, degradation of fibrinogen, haemoglobin, collagen and IgG, likely to have roles in feeding, attachment to the host as well as immunomodulation. Immunisation with AC-1, a 35 kDa secreted cysteine protease from *H. contortus*, protected against homologous challenge in sheep, reducing worm burden, fecundity and survival (Boisvenue *et al.*, 1992; Cox *et al.*, 1990). In vaccination studies with fractionated *H. contortus* extracts, it was found that only a fraction which was enriched for cysteine proteases conferred protection to lambs, resulting in a 47% decrease in worm burden and 77% reduction in faecal egg counts, suggesting that they are likely to be the immunodominant protective antigens for *H. contortus* infections (Knox *et al.*, 2005). Less experimentation has been done on protease inhibitors, but injection with anti-nippocystatin antibodies, an *N.*



*brasiliensis* secreted cysteine protease inhibitor which blocks host antigen-processing, resulted in reduced and earlier termination of egg production when challenged with *N. brasiliensis* in mice (Dainichi *et al.*, 2001b).

Glutathione S-transferases (GST) represent another class of nematode secreted proteins. One of its variants, *Hc*-GST-1 was identified from adult *H. contortus* cDNA library and characterised. It was found that that *Hc*-GST-1 bound haematin with high affinity, and it was postulated that it may be important for the detoxification or transport of heme, which holds particular significance to blood-feeding nematodes such as hookworms (Van Rossum *et al.*, 2004). Dogs vaccinated with *A. caninum* Ac-GST-1 show a 39.4% decrease in worm burden and 32.3% reduction in faecal egg counts during homologous challenge, although results did not reach statistical significance (Zhan *et al.*, 2005). However, it was shown in the same study that vaccination of hamsters with the same antigen resulted in a significant decrease in worm burden (53.7%) against heterologous challenge with *N. americanus*, so that the hookworm homologue of this protein (*Na*-GST-1) is now also being considered as a vaccine candidate by the HHVI (Periago, 2010; Sabin, 2011).

Earlier immunoblotting experiments with sera from sheep hyperimmune to *H. contortus* revealed two antigens at 15 and 24 kDa (named Hc15 and Hc24) which were strongly immunogenic, and immunisation with these purified antigens resulted in a 70% reduction in worm burden (Schallig *et al.*, 1997). Hc24 was subsequently found to be a homologue of the venom-allergen homologue / *Ancylostoma* secreted protein (ASP)-like (VAL) protein family through bioinformatic analyses (Cantacessi *et al.*, 2009). The VAL proteins are found in virtually all parasitic nematodes studied to date, and vaccination studies show that they are protective to many other parasitic nematode species including hookworms in dogs and humans (Bethony *et al.*, 2005). These targets were chosen as the first and only vaccine candidates targeting the larval forms of human hookworms by the HHVI to date (Bethony *et al.*, 2008b; Sabin, 2011). Findings in our laboratory indicate that there exist at least eight variants of VAL proteins in *N. brasiliensis*, and this will be discussed in Chapter 5.

Evidently, there have been few vaccine candidates studied to date which can confer 100% protection against parasitic nematodes. However, whether sterilising immunity

is needed is dependent on the application. A very high level of protection is probably more important for helminth control in livestock due to commercial reasons. On the other hand, this is not quite as critical in control of human hookworms. Unlike viruses or bacteria, helminths generally do not reproduce asexually in the host, and parasite burden increase mainly through reinfections. Therefore a partial reduction in worm burden and/or fecundity may still be able to significantly reduce the rate of transmission, morbidity and host pathology including anaemic and iron deficient conditions. This has been demonstrated using a computer simulation model for hookworm which evaluated the cost-effectiveness of vaccines (Lee *et al.*, 2011). The simulation programme indicated that a partial reduction in worm burden using a vaccine with an efficacy as low as 30% will still bring substantial health and economic benefits, particular to children and women of reproductive age, the population groups most susceptible to infection. It also indicated that optimal cost-effectiveness occurs when a combination of such a vaccine and current drug treatment is used for control of hookworms.

The ideal vaccine should also be able to protect against a number of helminth species. This is the appeal of investigating VALs, cysteine proteases and GSTs as vaccine candidates, as they are proteins commonly secreted by a number of helminth species. Although many therapeutic targets and vaccine candidates have been developed without extensive knowledge about their *in vivo* functions, it is still important to elucidate their role in host-parasite relations to avoid the evocation of undesired mechanisms and pathology in the host. Our major research interest lies in larval secreted proteins, as vaccines based on protective antigens found at the L3 stage may have the added advantage that intervention and vaccination strategies can be effective at a much earlier stage than when targeting proteins at the adult stage, thus minimising damage and suffering to the host.

## 1.8. Characterisation of secreted proteins in *N. brasiliensis* and other nematodes

The molecular and biochemical characterisation of the *N. brasiliensis* secretome is still in its infancy, mostly because a sequenced reference genome is not yet available for this organism. A major effort in this task has been provided by Marcus *et al.* (2004), who conducted a comprehensive bioinformatic analysis on the expressed sequence tags (ESTs) of *N. brasiliensis*. In this project, they utilised a full-length cDNA library constructed by oligo-capping to study sequences bearing signal peptide regions. They found that a high proportion (14.3%) of total ESTs have a conventional 5' signal peptide sequence, which suggest that they are likely to be secreted. The ESTs with signal sequences are less conserved with *C. elegans* or non-nematode genes than those without, suggesting that parasite secreted proteins may have experienced accelerated evolution compared to somatic proteins; that is, secreted proteins are likely to be particularly important for the parasite lifestyle. Many novel sequences which showed no homology to known proteins were found, and 32% of these have signal peptides, whereas only 3.4% of genes conserved to non-nematodes have signal peptides. This suggests that there is much room for exploration in the area of secreted proteins, as it is likely that the novel sequences may encode for yet undiscovered genes which are specifically important for the parasitic lifestyle. The EST sequences with homology to other nematode genes revealed that many of these code for proteases, lectins, VAL proteins and other enzymes, which is largely consistent with studies on secreted proteins of other nematode species.

Only a few specific secreted proteins have been characterised in *N. brasiliensis* to date. These include the acetylcholinesterases (AChE), the globins, a platelet activating factor acetylhydrolase (PAF-AH), and a cystatin. All of these proteins were discovered from the secreted products of adult stage *N. brasiliensis*, although we have recently demonstrated that AChE is also secreted by *N. brasiliensis* L3 upon activation (Huang *et al.*, 2010).

AChE is an enzyme which hydrolyses acetylcholine (ACh) to acetate and choline. Although its secretion has been found in many GI nematodes including *H. contortus*, *H. polygyrus*, *T. circumcincta*, *T. colubriformis* (the *Trichostrongylus* spp) (Ogilvie *et al.*, 1973), *N. americanus* (Pritchard *et al.*, 1991), *D. viviparus* (McKeand *et al.*, 1994)

and *A. ceylanicum* (Tekwani, 1992), their roles in the context of infection is yet unclear. The first evidence of AChE secretion was found in *N. brasiliensis* by Lee (1970) via cytochemical staining. This was then confirmed by Sanderson (1972), who found AChE activity in the secretory products of adults cultured *in vitro*. AChE was later purified from adult secreted products, and three isoforms (A, B and C) of this enzyme was found in *N. brasiliensis* which are distinguishable by non-denaturing polyacrylamide gel electrophoresis (PAGE) (Grigg *et al.*, 1997). All three isoforms are monomeric and hydrophilic. These proteins were later cloned and expressed in *Pichia pastoris*, and the recombinants show high and specific efficiency in hydrolysing acetylthiocholine, an ACh analogue (Hussein *et al.*, 1999). The isoforms A, B and C have apparent molecular weights of 74, 69 and 71 kDa respectively, and all of them have acidic isoelectric points (*pI*) (Hussein *et al.*, 2002; Hussein *et al.*, 2000). Isoforms B and C are 90% identical in amino acid sequence, whereas AChE A is only 63-64% identical to the others. The expression of these isoforms appears to be developmentally regulated, as AChE A is secreted by L4 immediately after its arrival into the duodenum, and AChE B and C are secreted when worms have matured and migrated to a more distal position in the jejunum (Blackburn and Selkirk, 1992a).

It is likely that AChE may contribute to some biological function to parasitic nematodes *in vivo*, as vaccination with AChE B in the rat resulted in reduced egg output of *N. brasiliensis* after a challenge infection (Ball *et al.*, 2007). Quantitatively, AChE production is positively correlated with immune pressure from the host (Selkirk *et al.*, 2005), but it is unclear whether this is caused by a general stress response or a functional role in immunomodulation. In earlier studies, it has been hypothesised that AChE may regulate contractile processes in the gut by inhibiting host ACh-induced peristalsis, thus protecting the worms from mechanical expulsion (Lee, 1970). Although *N. brasiliensis* adult secretory products were found to reduce the amplitude of contractions in rat intestines *in vitro*, administration of exogenous AChE did not produce the same effect, suggesting this phenomenon is likely to be regulated by other substances (Foster *et al.*, 1994). Another possibility is that AChE may play a part in inhibiting host ACh-induced mucosal fluidity, thus blocking the 'weep' in the 'weep and sweep' mode of nematode expulsion (Selkirk *et al.*, 2005). Indeed, ACh has been shown to stimulate exocytosis in enterocytes, Paneth cells, and epithelial granulocytes (Cooke, 2000; Satoh *et al.*, 1992). AChE purified from the

secretory products of *T. colubriformis* and *Nematodirus battus* was also shown to have direct modulatory effects on the proliferation of epithelial cells (Huby *et al.*, 1999). These studies suggest that AChE may possibly have a role in modifying the physiology of the host gut to enhance survival. On the other hand, the role of AChE may be unrelated to host physiology, for example it has been proposed that secretion of AChE into the environment directly exterior to the parasite may create a protective barrier to cholinesterase inhibitors ingested during feeding (Selkirk *et al.*, 2005), thus protecting the neuromuscular AChE within the worms which is important for neurotransmission.

*N. brasiliensis* expresses two forms of globins: an 18 kDa secreted / cuticular form, and a 17.5 kDa somatic form which lacks a signal peptide (Blaxter *et al.*, 1994). These globins have oxygen affinities which are a hundred-fold higher than the haemoglobins of their rodent host, although they share a common core globin domain containing a single haem group. Nematode globins are thought to be involved in the scavenging of oxygen, the supply of which is scarce within the gut environment. The expression of globins may thus provide a high-affinity oxygen uptake system for nematodes residing within the GI tract. Expression of the *N. brasiliensis* globins is developmentally regulated; the somatic form is first expressed upon host invasion, and the secreted globin is expressed exclusively by intestinal-dwelling adults. Globins are not expressed by resting L3 in the free-living phase, which presumably does not need to acquire extra oxygen in this manner. Globins have been found across all taxa of nematodes, suggesting that it is probably a protein common to all species (Blaxter, 1993). Nematode globins have been found to exist as monomers, dimers, trimers or tetramers, indicating that their quaternary structures are distinct to those of vertebrates. The ubiquity of globins however, makes them a potentially useful tool for phylogenetic analysis in inferring inter-species relationships.

Platelet activating factor (PAF) is a host-secreted phospholipid mediator which induces platelet aggregation, inflammation and anaphylaxis (Janeway *et al.*, 2005). It has been observed that adult secretory products of *N. brasiliensis* was able to inhibit PAF-induced platelet aggregation *in vitro* (Blackburn and Selkirk, 1992b). This activity was heat labile and specific for PAF, as the secretory products did not affect thrombin-induced platelet aggregation. The reaction was found to be effected by an

enzyme acetylhydrolase, which esterified an acetyl group on the glycerol backbone of PAF, thus inactivating its function. Further characterisation of this enzyme, a PAF-acetylhydrolase (PAF-AH), indicated that it exists as a heterodimer in native form, consisting of two protein subunits with apparent molecule masses of 38 and 25 kDa (Grigg *et al.*, 1996). As platelet aggregation and proinflammatory responses are generally unfavourable to the survival of GI nematodes, the secretion of PAF-AH by *N. brasiliensis* is likely to be a possible strategy in immunoevasion.

A 14 kDa cysteine protease inhibitor, named Nippocystatin, has been identified in *N. brasiliensis* adult secretory products, and was proposed to interfere with antigen-processing in APCs (Dainichi *et al.*, 2001b). As discussed, vaccination with this protein resulted in reduced egg production upon a challenge infection with *N. brasiliensis*. It is known that the activity of lysosomal proteases, including those of cysteine proteases, is important for the processing of pathogenic antigens in APCs prior to antigen-presentation via MHC (Janeway *et al.*, 2005). Nippocystatin was found to inhibit the activity of cysteine proteases specifically, and inhibited their cleavage of a third-party antigen, OVA, *in vitro* (Dainichi *et al.*, 2001b). The administration of Nippocystatin to mice decreased lysosomal cysteine protease activity *in vivo*, and co-immunisation of Nippocystatin with OVA reduced OVA-antigen-specific cellular proliferation in splenocytes, but not concanavalin A (ConA)-induced (non-antigen-specific) proliferation. Antigen-specific release of both IL-4 and IFN- $\gamma$  were reduced, suggesting a general suppression of T cell function. It was thus proposed that the secretion of Nippocystatin in *N. brasiliensis* may be utilised as a strategy in immunoevasion. Although Nippocystatin was identified in adult secretions, its mRNA was also expressed by activated L3 (Dainichi *et al.*, 2001a). Moreover, Nippocystatin also showed amino acid sequence homology to the cystatins of *C. elegans* and a range of filarial nematodes, in which signature sequences of the cystatin superfamily were conserved. Although filarial cystatins have also been shown to inhibit T cell proliferation, their mode of action may differ to that in Nippocystatin, as they were shown to inhibit ConA or anti-CD3-induced (non-antigen-specific) T cell proliferation in general and also stimulated production of the immunosuppressive cytokine IL-10 (Hartmann *et al.*, 1997; Hartmann and Lucius, 2003; McKerrow *et al.*, 2006).

Although a few *N. brasiliensis* secreted proteins have been studied in some detail, there is still, evidently, a lack of breadth in knowledge, particularly for proteins secreted by the larval stages. This observation was common in many other nematode species, but much progress has been made in the last decade as the availability of genome information increased and high-throughput proteomic methods were developed. The adult secretomes of *H. contortus* (Yatsuda *et al.*, 2003), *A. caninum* (Mulvenna *et al.*, 2008) and *B. malayi* (Hewitson *et al.*, 2008) have been analysed by large-scale proteomic analyses. These studies provided a suite of newly identified proteins which were not previously known to contribute to the parasitic lifestyle, and also revealed factors which are commonly secreted by many parasitic nematodes, such as proteases, protease inhibitors, lectins, VAL homologues, antioxidants, as well as certain enzymes and immunomodulatory molecules, etc. Although such studies have greatly improved our understanding of nematode secretions, proteins secreted by different life cycle stages are likely to vary accordingly to the challenges the parasites face at different points during the infective process. This has been demonstrated in a preliminary proteomic survey sampling secreted proteins from L4 and adults of *T. circumcincta* (Craig *et al.*, 2006). Stage- and gender-specific secreted proteins have also been studied in *S. ratti* (Soblik, 2009) and *B. malayi* (Moreno and Geary, 2008), which again were shown to be unequivocal. Changes which occur at the point at which resting larvae transits from its free-living existence to the parasitic phase are probably the most exciting and informative, and this has been studied in *A. caninum* by transcriptional profiling, which revealed a large range of differentially transcribed mRNA between activated and non-activated L3 (Datu *et al.*, 2008). Partial proteomic characterisations were also carried out for proteins secreted by muscle-stage larvae of *T. spiralis* (Robinson and Connolly, 2005) and mucosal-stage larvae of *T. circumcincta* harvested early post-infection (Smith *et al.*, 2009). These data will be useful in inferring host-parasite relationships as the identified proteins are functionally characterised. Thus studying proteins secreted by activated larvae would highlight molecules which are particularly important for the infective process and the parasitic lifestyle, which may reflect the strategies required to establish host-parasite relationships in a myriad of extraordinary ways.

## 1.9. Summary and aims

It is clear that secreted proteins are important for the parasitic lifestyle, with potential functions ranging from invasion, establishment of infection, feeding, and modulation of the host immune response. Using *N. brasiliensis* as a model, the aim of this project is to improve our understanding of nematode secretions, with particular focus on larval secreted proteins.

### **Aim 1: To analyse *N. brasiliensis* secreted proteins by two-dimensional gel electrophoresis (2-DE)**

Proteins secreted by the larval stage of most parasitic nematodes has been a topic much neglected, probably due to a lack of knowledge of the factors required to activate secretion, and the fact that they secrete very small amounts of proteins. One of the major aims is to characterise the secretion profile of *N. brasiliensis* larvae by 2-DE. The first tasks are to collect sufficient material analysis by routine cultivation of large batches of larvae, and to optimise electrophoretic conditions. Although the major interest of this project is in larval secreted proteins, adult secreted proteins will also be analysed in comparison. Since *N. brasiliensis* is not a genome-verified organism, the global proteomic identification of all secreted proteins is not a major aim.

### **Aim 2: To compare two strains of *N. brasiliensis* in terms of infection dynamics, host response and secreted proteins**

At the beginning of this project, a field strain of *N. brasiliensis* was donated to our laboratory by Professor Mark Viney (University of Bristol, UK). This strain was noticeably more productive than our laboratory strain, as observed from initial life cycle propagation. Another aim of this project is thus to compare the infection dynamics of the two strains and the host responses they elicit. This study may illustrate the effects of laboratory passage on properties of the parasite. Importantly, if the two strains differ in infectivity, this would provide a classifier to distinguish factors important for the infective process, in which case potential differences seen in larval secreted proteins of the two strains will be analysed.



**Aim 3: To investigate the activation of larval protein secretion**

The L3 stage of *N. brasiliensis* and many other GI nematodes are developmentally arrested during the free-living phase, until host encounter triggers changes in the worms which transits them into their parasitic phase. This process is known as ‘activation’, which is central to the infective process. By mimicking host-like factors, their effect on larval activation and protein secretion will be investigated.

**Aim 4: To investigate the effect of immunisation with a larval secreted protein**

One of the ultimate goals of studying nematode secreted proteins is to discover molecules which can confer protective immunity through vaccination. A major aim of this project is thus to perform vaccination trials with a candidate protein secreted by *N. brasiliensis* larvae. The VAL proteins were initially found to be abundantly secreted by *A. caninum* larvae after activation by host-like factors, suggesting that they are likely to be molecules involved in the infective process. Characterisation of VAL proteins has been an ongoing project in our laboratory, and one of my major aims is to perform vaccination trials with a larval-secreted VAL protein.

# **Chapter 2**

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## **Materials and Methods**

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## 2.1. Infection and recovery of *N. brasiliensis*

The life cycle of *N. brasiliensis* was maintained in male Sprague-Dawley (SD) rats, housed in the Central Biomedical Services at Imperial College London. To establish an infection, SD rats were subcutaneously injected with infective larvae (L3) in phosphate buffered saline (PBS), typically at a dose of 7,000 per rat for the Wellcome strain, and 2,000 for the Japanese strain, unless otherwise stated. Rat faeces harbouring eggs of the parasite were collected from 6 to 10 days post-infection, hydrated with distilled water, mixed with charcoal and incubated at room temperature for a minimum of one week to allow eggs to hatch and larvae to moult into L3. Experimental infections of mice were carried out at 500-600 L3 per mouse.

L3 were recovered from week-old faeces by filtration in a Baermann apparatus with water at 20°C through 3 layers of muslin and 2 layers of lens tissue for 90-120 minutes. Recovered L3 were collected into a 15 ml Falcon tube and washed extensively with PBS. The number of recovered L3 was estimated by counting viable larvae on an egg counting chamber (McMaster).

L4 can be collected from the lungs of rats or mice at 24 to 48 hours post-infection. The lungs were harvested by dissection of the thoracic cavity, then placed on a small piece of muslin on a small weighting boat, where they were minced finely with dissection scissors and sharp forceps. A solution of 1% agarose in PBS kept at 37°C was added to the weighting boat (5 ml per set of lungs) and left to set. The solidified mixture was lifted from the weighting boat from an exposed edge of the muslin, then entirely wrapped in a fresh 7 x 7 cm muslin piece and secured at the top of a 50 ml falcon tube containing PBS at 37°C. The tubes were incubated overnight at 37°C, during which L4 worms migrate out of the tissue and agarose. L4 were then collected at the bottom of the tube and counted under a light microscope.

To collect adult stage *N. brasiliensis* worms, infected rats or mice were sacrificed on day 4-10 or day 4-7 post-infection respectively. The intestines of the rats were harvested and cut opened by longitudinal incision. Adult parasites were recovered by incubating the dissected rat intestine in PBS at 37°C for 1-2 hours whilst filtering through one layer of muslin.

## **2.2. *In vitro* cultivation of *N. brasiliensis***

Prior to cultivation, L3 were further sterilised from bacteria and fungi by incubation for 30 minutes to 1 hour in PBS supplemented with 1000 U/ml penicillin, 1 mg/ml streptomycin, 200 µg/ml gentamicin and 200 U/ml nystatin. They were then washed and resuspended in worm medium (WM; 1% (w/v) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, 20 U/ml nystatin, 2 mM L-glutamine in RPMI-1640). L3 were normally cultured at approximately 100,000 worms per 10 ml WM at 37°C in 5% CO<sub>2</sub> for a period of three days, unless otherwise stated. The first 24 hours of cultivation is considered an 'activation period'.

Adult worms were washed extensively with PBS and at least three times with WM prior to culture. Adults were typically cultured at 2,000 worms per 10 ml medium at 37°C in 5% CO<sub>2</sub> for a period of 3 days. The culture medium was collected and changed each day during the cultivation period.

## **2.3. Radioactive labelling of secreted proteins**

Since L3 secrete only very small amounts of proteins, radioactive labelling allows for a more sensitive method of detection during the initial assessment of their secretion profiles. In addition, the effect of other factors such as serum or rat skin extracts on the secretion profiles of L3 can also be determined. Secreted proteins which are newly synthesised by the parasites during cultivation were radioactively labelled by the addition of 0.2 mCi of <sup>35</sup>S-methionine (<sup>35</sup>S-Met) per ml of WM during the cultivation period, at 20°C or 37°C. For serum or rat skin-induced activation conditions, WM was supplemented with 10% rat serum or rat skin fractions (extracted from 0.25 g of skin per ml WM, described in section 2.18) during the activation period at 20°C or 37°C, after which L3s were washed extensively with WM before re-incubation with fresh WM with <sup>35</sup>S-Met for the remaining 48 hours of culture. The amount of protein in <sup>35</sup>S-Met incorporated preparations was determined by liquid scintillation counting.

For analysis of pre-synthesised proteins, L3 were pre-incubated at 20°C for 40 hours with 0.25 mCi of <sup>35</sup>S-Met per ml of WM. After this initial labelling, L3 were washed

thoroughly to eliminate the remaining  $^{35}\text{S}$ -Met in the medium. The washed L3 were then resuspended in 1 ml of fresh worm medium for each condition and stimulated with the aqueous or lipid fractions of rat skin extract (each equivalent to fractions extracted from 0.25 g of rat skin) for 30 minutes at 20°C or 37°C, after which the culture medium was collected.

#### **2.4. Recovery and concentration of secreted products**

At the end of the cultivation period, the worms were left to settle to separate them from the medium. The culture medium containing secreted products was collected and sterilised by filtering through a 0.2  $\mu\text{m}$  syringe filter (Sartorius) and stored at -20°C until further use. Unless otherwise stated, the medium was pooled from at least 6 individual batches of culture. Small and large volumes of media were concentrated using centricon and amicon ultrafiltration units (Millipore), with a 3 kDa molecular weight cut-off membrane, respectively. Concentrated samples were buffer exchanged at least three times with 25 mM hepes (pH 7.5) or PBS (pH 7.4) to eliminate WM components to trace levels.

#### **2.5. Assessment of protein concentration**

Protein concentration was determined using the bicinchoninic acid method (Pierce) according to the manufacturer's instructions. Bovine serum albumin (BSA) ranging from 0.125 mg/ml to 1.5 mg/ml was used as standards.

#### **2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein samples were separated using polyacrylamide gels consisting of a 5% stacking gel (5% (v/v) acrylamide mix, 0.125 M Tris base pH 6.8, 0.1% (w/v) SDS, 0.15% (w/v) ammonium persulphate and 0.1% (v/v) TEMED) and a 10%, 12% or 15% separating gel (10%, 12% or 15% (v/v) acrylamide mix in 0.4 M Tris base pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.04% (v/v) TEMED),

depending on the complexity and molecular weight range of the sample. Gradient gels were purchased from Bio-rad (Criterion Tris-HCl gels, 10-20%). Protein samples were incubated at 95°C for 10 minutes with a protein loading buffer (0.2 M sucrose, 6% (w/v) SDS, 125 mM Tris base pH 6.9, 4 mM EDTA, 2% (v/v) β-mercaptoethanol and 0.001% (v/v) bromophenol blue) prior to loading. Samples were separated on the gel in SDS-PAGE running buffer (25 mM Tris-Cl pH 8.3, 192 mM glycine and 0.1% (w/v) SDS) at a constant current of 30 mA and a maximum voltage of 150 V, until the leading edge of the dye has reached the bottom of the gel. The unstained precision plus protein standard (Biorad) was typically used as a molecular weight marker. Gels were stained overnight at room temperature (RT) in 0.4% (w/v) Coomassie brilliant blue stain in 10% (v/v) acetic acid and 40% (v/v) methanol. The gels were destained with 10% (v/v) acetic acid and 40% (v/v) methanol until protein bands could be clearly visualised.

## **2.7. Two-dimensional gel electrophoresis (2-DE)**

The 2-DE method separates proteins on both a charge and mass basis. Protein samples were prepared using acetone precipitation. Cold acetone (-20°C) and the protein sample were mixed in a 4:1 volume ratio and incubated overnight at -20°C. The sample was then centrifuged at 16,000 x g for 30 minutes at 4 °C. The supernatant was discarded and the pellet was allowed to air-dry for 15-30 minutes until all traces of acetone had evaporated. Rehydration buffer (8 M urea, 2% (w/v) 3-3'-(Cholamidopropyl)-3,3-dimethylammoniumpropylsulfate (CHAPS), 0.5% (v/v) IPG buffer, 0.002% bromophenol blue in ddH<sub>2</sub>O) at a volume of 100 µl was added to the precipitate and vortexed thoroughly until the pellet had dissolved.

The protein sample was subjected to a first-dimensional separation by isoelectric focusing (IEF) on immobilised pH gradient gels (IPG) strips (11 cm, 3-11NL, GE Healthcare), carried out on the IPGphorIII IEF machine (Amersham). The sample dissolved in rehydration buffer was dotted along the chamber of the IPG strip holder in droplets and the IPG strip was laid on top whilst avoiding any bubbles. The chamber was filled with 1 ml of cover fluid (GE Healthcare) to prevent evaporation, closed and aligned along the electrodes in the IEF machine. The optimised

programme was as follows: rehydration for 12 hours at 30 V, then 1 hour at 500 V, 1 hour at 1000 V, and a final focusing at 8000 V for 5 hours. An equilibration buffer (EqB) was prepared (50 mM Tris-Cl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS in distilled water). The IPG strip was subjected to a first equilibration in EqB with 0.01 g/ml dithiothreitol (DTT), and a second equilibration in EqB with 0.025 g/ml iodoacetamine. For the second dimensional separation, the strip was transferred to 10-20% gradient precast gels (Biorad) and vertically resolved at 40 V for 10 minutes, then at 150 V for 100 minutes. The unstained precision plus protein standard (Biorad) was used as a molecular weight marker.

## **2.8. Autoradiography**

For radioactively labelled samples, separated bands or spots on the gel were visualised by autoradiography. After electrophoresis, polyacrylamide gels were dried and exposed to X-ray film (Fuji) in lightproof cassettes with intensifying screens for a length of time as appropriate to the amount of radioactive sample loaded. The cassettes were incubated in a -80°C freezer to obtain a more refined exposure for <sup>35</sup>S-incorporated samples, and the films were developed in the darkroom.

## **2.9. Molecular comparison of parasite strains**

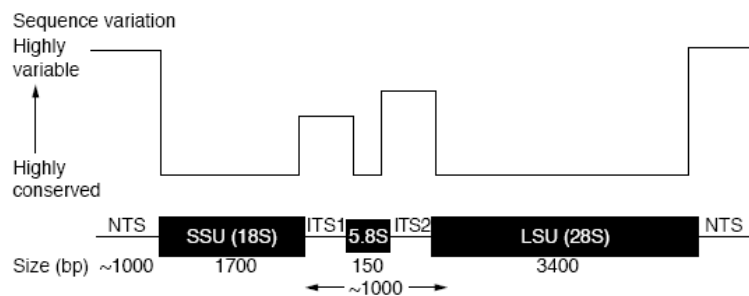
Genomic DNA (gDNA) was obtained from single adult parasites. A master mix of worm lysis buffer (WLB) was prepared (110 mM NaCl, 110 mM Tris-Cl pH 8.5, 55 mM EDTA, 1.1% (w/v) SDS, 1.1% (v/v) 2-mercaptoethanol). Immediately before use, proteinase K was added to WLB at a final concentration of 100 µg/ml, and 10 µl of this mixture was added to each individual worm in separate PCR tubes. The tubes were frozen at -80°C overnight. The following day, the frozen tubes were transferred to a pre-heated heat block at 65°C for 1 hour followed by 5 minutes at 95°C. This extract was used as the PCR template at a 1 in 100 dilution.

For a molecular analysis of the two strains of *N. brasiliensis*, regions within the ribosomal RNA gene (rDNA) were amplified by polymerase chain reaction (PCR).

Regions selected for comparison include the conserved small subunit (SSU) gene and the variable internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) (Fig. 2.1). The primer sequences used to amplify these regions are shown in Table 2.1. The PCR reactions consist of 500 nM of the forward and reverse primers, 250  $\mu$ M of each dNTP (dATP, dTTP, dCTP and dGTP), 25 mU/ $\mu$ l *Taq* DNA polymerase, and 2  $\mu$ l of the PCR template constituted in standard *Taq* buffer (B9014S, NEB) to a final volume of 40  $\mu$ l. The amplification conditions used were 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 1 minute, and a final extension for 10 minutes at 72°C. PCR products were resolved on a 1.5% (w/v) agarose gel in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate, 1 mM EDTA) with inclusion of 0.5  $\mu$ g/ml ethidium bromide (EtBr) in the gel for visualisation of DNA bands under ultraviolet (UV) light. Successfully amplified PCR products were purified using the Qiaquick PCR purification kit (Qiagen) according to manufacturer's instructions, and the DNA concentration and purity were assessed photospectrometrically. Each purified sample was sent for automated sequencing with the forward primers for SSU, ITS-1 and ITS-2 at Cogenics (Beckman Coulter Genomics). Each sequence was obtained from 3 individual worms from each strain, and their alignments were verified to be identical. Sequences from the two strains were aligned using ClustalW and Multialign analyses, then shaded and presented using the Boxshade Server 3.21.

The sequences of ITS-1 and ITS-2 were aligned with those of other parasitic nematodes closely related to *N. brasiliensis* to construct a phylogenetic tree using the neighbour joining method. ITS-1/ITS-2 sequences were retrieved from Genbank (NCBI) for 7 parasitic nematodes within the order Strongylida (Table 2.2). The alignment matrices for ITS-1 and ITS-2 were concatenated within the Mesquite system for phylogenetic computing (Maddison and Maddison, 2010), and phylogenetic analyses was carried out on Phylogeny.fr (Dereeper *et al.*, 2008), utilising algorithms based on the neighbour-joining (NJ) method to determine phylogenetic distances, then verified using maximum likelihood (ML) analysis.





**Figure 2.1. Structure of the rRNA cistron**, diagram from Dorris *et al.* (1999). SSU, small subunit gene; ITS, internal transcribed region. Sequence variability of regions of the gene within the phylum Nematoda are shown above the gene structure.

Target	Primer name	Sequence 5' to 3'
SSU	SSU18A (F)	AAAGATTAAGCCATGCATG
	SSU26R (R)	CATTCTTGGCAAATGCTTTCG
ITS-1	NC5_F (F)	GTAGGTGAACCTGCGGAAGGATCATT
	NC13_R (R)	GCTGCGTTCTTCATCGAT
ITS-2	ITS2F (F)	CAGTGGGGCTTGTAGTACAC
	ITS2R (R)	CACAAACAAGCGGTACCATTATG

**Table 2.1. Oligonucleotide primer sequences for PCR amplification of the SSU, ITS-1 and ITS-2 regions.** F, forward primer; R, reverse primer.

Species	Accession number	
	ITS-1	ITS-2
<i>C. minutus</i>	AY332645.1	AY333379.1
<i>O. erbaevae</i>	AY332647.1	AY333381.1
<i>H. polygyrus</i>	AY332649.1	AY333382.1
<i>N. battus</i>	AJ251569.1	Y14010.1
<i>N. filicollis</i>	AJ251572.1	Y14011.1
<i>H. contortus</i>	AF044927	X78803.1
<i>T. circumcincta</i>	AF044934.1	X86026.1

**Table 2.2. Genbank accession numbers for ITS-1 and ITS-2 sequences of parasitic nematode species within the order Strongylida.**

## **2.10. Faecal egg count**

The egg output of intestinal nematodes is an important determinant of infection intensity, and a salt-flotation method was employed to isolate parasite eggs from faeces. Rats or mice were caged individually, and all faecal pellets produced by each animal were collected every 24 hours into a plastic packet. Each packet of faeces were soaked in distilled water at 10 ml/g faeces and broken up into a suspension. 10 ml of the faecal suspension was withdrawn and centrifuged at 100 x g for 2 minutes to sediment faecal material and eggs. The pellet was resuspended in 10 ml of saturated sodium chloride (NaCl) and centrifuged for another 2 minutes at 100 x g. At this point the eggs are floating at the meniscus. The top 1 ml of the supernatant was withdrawn and the eggs within were counted in a haemocytometer. The number of eggs in a particular sample was expressed as number of eggs per gram (epg) of dry faeces collected.

## **2.11. *In vitro* egg counts**

Adult worms were cultured *in vitro*, and the number of eggs released during the cultivation period was used as a measure of fecundity. Adults were cultured at 37°C, 5% CO<sub>2</sub> at 2,000 adults per group, in 10 ml WM, for a period of 24 hours. The number of eggs released was quantified using a McMaster egg counting chamber and divided by the number of adult worms in culture.

## **2.12. Histology of rat jejunum**

Sections of jejunum were harvested from sacrificed rats and prepared for histology. Jejunum portions (1.5 cm) were cut at approximately 30 cm distal from the pyloric sphincter, and gut contents were flushed out gently with PBS using a narrow-tip glass Pasteur pipette. The tissue was fixed in neutral buffered formalin (NBF), embedded in wax, cut in cross-section and stained with Periodic acid-Schiff (PAS) for visualisation (histology performed by Miss Lorraine Lawrence, NHLI, Imperial College London). Goblet cells were stained magenta due to their rich carbohydrate content, and were

enumerated per 20 villus crypt units. Mucosal depth, crypt length, and muscle thickness were measured using the LuciaG Laboratory imaging software (Nikon) and recorded.

### **2.13. Measurement of rat mast cell protease II (RMCP II)**

Levels of RMCP II in rat sera were assayed by enzyme-linked immunosorbent assay (ELISA) using the RMCP II ELISA kit (Moredun Scientific Limited, gift from Professor Dave Knox, Moredun Institute) according to the manufacturer's instructions. General procedures for ELISA are provided in section 2.24.

### **2.14. Lymphocyte proliferation and cytokine measurements**

Mesenteric lymph nodes (MLNs) were harvested from sacrificed rats and passed through a 100 µm cell strainer into a sterile tube. The cells were washed with RPMI-1640 and centrifuged at 300 x g at 4°C, then washed twice with cold medium. The cells were resuspended in cell culture medium (RPMI-1640 supplemented with 10% (v/v) foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin), counted, and seeded at 5 x 10<sup>5</sup> cells per well in a 96-well plate. *N. brasiliensis* adult worm homogenate (50 µg/ml) was added to the culture medium to a final volume of 200 µl/well for assessment of antigen-specific proliferation. The cells were incubated for 48 hours at 37°C and 5% CO<sub>2</sub>. At the end of the 48-hour incubation, the cell supernatant was collected for assessment of cytokine levels. Levels of IL-4, IL-5, IL-10, IL-12, IL-13 and IFNγ were assayed by using a 6-plex cytokine bead assay kit (Millipore) according to the manufacturer's instructions.

After the spent media was collected for cytokine measurements, the wells were refilled with culture medium with <sup>3</sup>H-thymidine added at 1 µCi per well, and the cells were incubated for a further 16 hours at 37°C and 5% CO<sub>2</sub> for assessment of lymphocyte proliferation. Cells were collected onto a filter disc using an automated 96-well plate cell harvester, after which radioactive counts (cpm) were assessed in a beta-counter.

## **2.15. Gel separation of acetylcholinesterase (AChE) isoforms**

A direct-colouring thiocholine method for cholinesterases was used to visualise isoforms of AChE on a 8% native polyacrylamide gel (Karnovsky and Roots, 1964). Adult secreted proteins were resolved on the native gel (same constitutions as 8% SDS-PAGE but with omission of SDS in all reagents) at 4°C until the dye front has reached two-thirds of its full length, then incubated in 65 ml of 0.1 M sodium or potassium phosphate buffer (NaPO<sub>4</sub> or KPO<sub>4</sub>), pH 6.5 at room temperature for 30 minutes. On a shaking platform, the following reagents were added sequentially: 50 mg acetylthiocholine iodide powder, 5 ml of 0.1 M sodium citrate, 10 ml of 30 mM CuSO<sub>4</sub>, 10 ml dH<sub>2</sub>O, and finally 10 ml of 5 mM potassium ferricyanide. The cholinesterases liberate thiocholine, which reduces ferricyanide to ferrocyanide. The latter combines with Cu<sup>2+</sup> ions to form an insoluble brown copper ferrocyanide. Excess Cu<sup>2+</sup> ions in the medium are complexed with citrate to prevent formation of copper ferricyanide. Bands stained for AChE were developed within 15-30 minutes as reddish-brown precipitations. The reaction was stopped in H<sub>2</sub>O and fixed in 10% acetic acid.

## **2.16. Measurement of AChE activity in secreted proteins**

The AChE activity of parasite secreted proteins was measured by using acetylthiocholine (ASCh) iodide, a hydrolysable analogue of acetylcholine (ACh), as substrate. In a 200 µl reaction on a 96-well plate, 0.5 µg of secreted proteins were added to the substrate mixture (1 mM ASCh iodide, 1 mM 5.5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 100 mM sodium phosphate, pH 7.0). The rate of hydrolysis of ASCh is similar to that of ACh, but reaction with the former results in liberation of thiocholine, which reacts with DTNB to give the yellow anion of 5-thio-2-nitrobenzoic acid. Absorbance was monitored colourimetrically at 414 nm and measured at 10 minutes (end-point) into the reaction. Results were presented as ΔOD per minute per µg protein.

### **2.17. Enzymatic assay for phosphate hydrolysis**

Nucleotide hydrolysing activities of *N. brasiliensis* adult secreted proteins were measured by assaying the amount of inorganic phosphate liberated in the reaction. In a total volume of 40 µl, reactions were carried out in a buffer consisting of 25 mM Hepes (pH 7.50), 150 mM sodium chloride (NaCl), 5 mM magnesium chloride (MgCl<sub>2</sub>) and 2.5 mM dithiothreitol (DTT). The reactions were initiated by the addition of *N. brasiliensis* adult secreted products (protein concentration ranging from 0.15 to 5 µg in 25 mM hepes, pH 7.50) and an appropriate nucleotide substrate (ATP, ADP, UDP or AMP) at a final concentration of 2.5 mM. Controls for spontaneous or nonenzymatic phosphate release from nucleotides and secreted proteins were assayed in parallel. All reactions were incubated at 37°C for 5 to 10 minutes, after which 200 µl of the chromogenic reagent malachite-green phosphomolybdate (1.25 mM malachite green, 8.6 mM ammonium phosphomolybdate in 1.7 N HCl) was added immediately to each reaction. The amount of liberated inorganic phosphate was measured by determining the absorbance at 600 nm, and quantified by comparison to a standard curve obtained with sodium dihydrogen orthophosphate (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O) at a range of 0.05-0.5 mM. The level of phosphatase specific activity was expressed as ng phosphate released per minute per mg of secreted protein (ng/min/mg protein).

### **2.18. Preparation of rat skin extracts**

Rat skin was collected from euthanised and shaved SD rats and fractionated into an aqueous and a lipid fraction as described in Safer *et al.* (2007), utilising the Folch method (Folch *et al.*, 1957). The skin of the rats was excised from the abdomen and the back of the rats, which is then cut and ground into a fine paste. Portions (2.2 g each) of the paste were extracted with 10 ml chloroform / methanol / water at a ratio of 2:1:0.2 (vol/vol) in sterilised glass tubes. The tube contents were mixed thoroughly, placed on a rotary mixer for 90 minutes, then centrifuged at 1,500 x g for 15 minutes to accelerate phase separation. This resulted in solvent partition into two distinct phases. The lower phase consists of chloroform / methanol / water at a ratio of 86/14/1, in which all lipids (polar and non-polar, irrespective of chain length) can be

solubilised (Folch *et al.*, 1957). The upper phase consists of chloroform / methanol / water at a ratio of 3/48/47, and contains the aqueous fraction, which was carefully extracted from the top without disturbing the phases. This aqueous phase was partially dried by rotary evaporation for 1-2 hours to remove residual organic solvent, and lyophilised to dryness. Lyophilised material was reconstituted with 1 ml of distilled water per 7.5 g of skin paste, resulting in an extract with a protein concentration of approximately 3 mg/ml. The organic phase containing the lipids was extracted from the bottom layer, transferred to a clean glass tube and dried down by nitrogen-facilitated evaporation. The resulting dried lipid was also resuspended with 1 ml of distilled water per 7.5 g of skin paste, and sonicated for 5 x 30 s before use.

### **2.19. Chemotaxis Assay for L3**

Chemotaxis assays (modified from Safer *et al.*, 2007) were performed in 60 x 15 mm sterile plastic Petri dishes containing 5 ml of 0.9% (w/v) agarose (unless otherwise stated), on a levelled platform at room temperature, in triplicates for each condition. A well measuring 8 mm in diameter and approximately 2 mm in depth was made at each end of the plate using a P1000 pipette tip and a scalpel, without disturbing the bottom of the agarose gel. On each plate, 50 µl of either test or control substance was pipetted into the wells, and allowed to diffuse into the agarose for 45 minutes. Thereafter, L3 (2 groups of 150 larvae per plate) suspended in 12 µl of PBS were added across the surface diameter, halfway between the two wells, in small droplets without disturbing the gel surface. The plates were placed in random orientations in a uniformly-lit room to allow L3 to disperse at will. The number of larvae at each well was scored every 30 minutes for 3 hours. The level of directed positive chemotaxis was determined by subtracting the number of L3 in the negative from the positive well. The test substances used in this assay include rat skin extracts (prepared as described in section 2.18), rat serum (Sigma-Aldrich), BSA (Roche), and urocanic acid (Acros Organics) at various concentrations. To test the effect of resistin-like molecules (RELMs) on chemotactic functions, L3 were pre-incubated with RELM $\alpha/\beta/\gamma$  (Peptotech) at 5 µg/ml or 50 µg/ml for 1 hour prior to performing the chemotactic assays. An additional control was introduced by pre-incubating a group of L3 with BSA in parallel.

## **2.20. Feeding assay with fluorescent BSA**

Fluorescent feeding assays were carried out at 20°C or 37°C to assess the activation of feeding in *N. brasiliensis* L3. Where indicated, 10% rat serum or fractions of rat skin (extracted from 0.25 g rat skin per ml of medium) were included in the worm culture medium (WM). In one of the specified treatment groups, L3 were chemically exsheathed by incubation with sodium hypochlorite (NaClO) in PBS for 20 minutes and washed six times prior to incubation.

Groups of approximately 2,000 L3 were sampled for feeding activation by the addition of fluorescein-5-isothiocyanate conjugated BSA (FITC-BSA) to a final concentration of 2.5 mg/ml during the final hour of incubation, after which they were washed four times with PBS and examined by fluorescence microscopy. A minimum of 200 viable larvae were counted from each of three triplicates per treatment group.

## **2.21. Feeding assay with radiolabelled BSA**

BSA was radiolabelled by the addition of 250 µCi Sodium-<sup>125</sup>Iodide (Na-<sup>125</sup>I, MP Biomedicals) to 50 µl of 1 mg/ml BSA in PBS, followed by 5 µl of 1 mg/ml Chloramine T to initiate iodination. The mixture was left to react for 2 minutes, after which another 5 µl of Chloramine T was added. After a further 5 minutes, the reaction was quenched by the addition of 10% (vol/vol) of saturated tyrosine solution. The products were then separated on a Sephadex G-25 column and eluted in 250 µl fractions. The level of <sup>125</sup>I-incorporated BSA in each fraction was tested by trichloroacetic acid (TCA) precipitation. Briefly, 1 ml of cold 10% TCA solution was added to 5 µl of sample and mixed thoroughly then incubated overnight at 4°C. The tubes were centrifuged at 13,000 x g at 4°C for 10 minutes, and the supernatant was discarded. The pellet was washed twice with 200 µl cold acetone and resuspended in PBS. Incorporated <sup>125</sup>I in each sample was measured in a gamma counter.

L3A or adult worms were fed with <sup>125</sup>I-BSA to assess their rate of feeding quantitatively. Groups of 4,000 L3A (activated by cultivation at 37°C for 24 hours) or

50 adults were incubated with  $2 \times 10^6$  cpm of  $^{125}\text{I}$ -BSA in 500  $\mu\text{l}$  of WM for 2 hours at 37°C. The worms were washed 6 times with PBS and transferred to a fresh tube, then counted for each of three replicates per treatment group measured in a gamma counter. Background levels were established by performing the assay in parallel on dead parasites killed by heating at 65°C for 10 minutes in triplicate.

## **2.22. Western blotting**

Proteins separated by SDS-PAGE or 2-DE were transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences) by Western blotting. Prior to transfer, the polyacrylamide gel, nitrocellulose membrane, filter paper and fibre pads were equilibrated in chilled transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol) at 4°C for 5 minutes, then arranged in a blotting cassette with the gel facing the cathode and the membrane facing the anode, with 4 sheets of filter paper and 1 fibre pad on each side. The Bio-rad mini or regular Tran-blot electrophoretic transfer cell was used for small or large gels, run at 90 V for 2 hours or 12 V overnight, respectively. Following electrophoretic transfer of protein, blots were visualised with DB71 staining to check for successful transfer, scanned and destained (Section 2.23). The blots were incubated on a shaking platform at RT for 1 hour in blocking solution (5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 in PBS) at RT, then incubated with the primary antibody diluted to an appropriate concentration with blocking solution for 2 hours or overnight at 4°C. The blots were washed 3 times with PBS-Tween (0.1% Tween-20 in PBS) for 10 minutes each. Secondary antibody conjugated to peroxidase was diluted to an appropriate concentration with PBS-Tween, and incubated with the blot for 2 hours at RT. Dependent on the species in which the primary antibody was raised, the secondary antibodies goat anti-rat IgG-HRP (Bio-rad) and goat anti-mouse IgG-HRP (Sigma) were typically used at a dilution of 1:60,000 and 1:2,000 respectively. The blots were then washed 3 times in PBS-Tween, and 3 times in PBS for 10 minutes each. Bound antibodies were detected using enhanced chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare). The blot was immersed in ECL reagent for 2 minutes, during which the peroxidase-catalysed oxidation of luminol (and subsequently enhanced chemiluminescence) was elicited on areas where the



peroxidase-conjugated antibody is bound to the antigen on the membrane. The resulting light is detected on Fujifilm superRX and developed in the darkroom.

### **2.23. DB71 staining of Western blots**

After protein transfer, blots were incubated in direct blue 71 (DB71) stain solution (0.008% DB71 in 40% ethanol, 10% acetic acid) for 5 minutes while protein bands or spots become visible. The stained blots were washed briefly in 40% ethanol, 10% acetic acid, then scanned to record the images in their original sizes. The blots were destained in 150 mM sodium bicarbonate in 47.5% ethanol prior to the next incubation steps in Western blotting.

### **2.24. Serum ELISA**

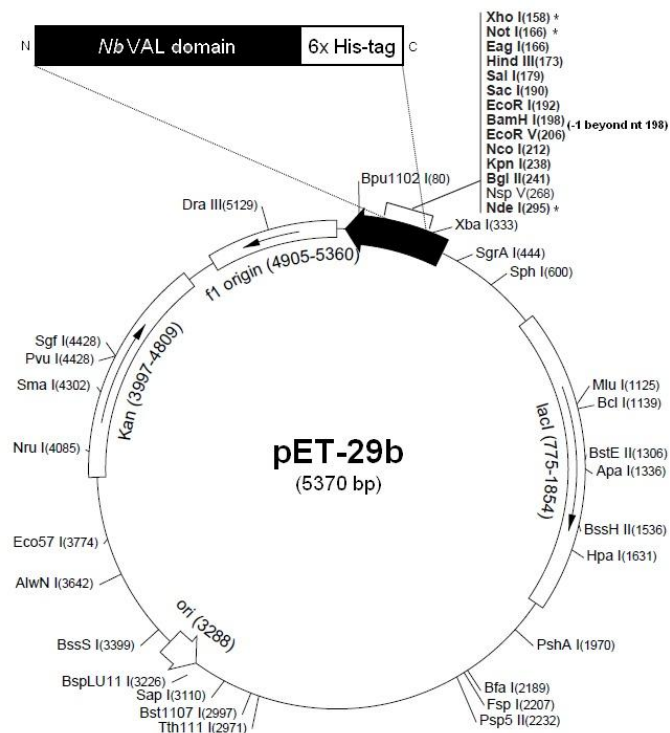
Antibodies to *N. brasiliensis* secreted proteins and *NbVALs* were detected by ELISA. Mice were bled by cardiac puncture or tail-vein bleed, and the collected blood was left to clot overnight at 4°C. The blood was centrifuged at 16,000 x *g* for 10 minutes, after which the serum was collected.

Maxisorb Surface 96-well plates (Nunc) were coated with 100 µl/well of antigen samples diluted to 5 µg/ml in coating buffer (0.06 M sodium carbonate buffer, pH 9.5). Plates were sealed and incubated overnight at 4°C. The coating antigen was then aspirated and the plates were washed 3 times with wash buffer (0.05% (v/v) Tween 20 in PBS). Plates were then incubated with 200 µl/well of blocking buffer (5% (v/v) FCS in PBS) for 2 hours at room temperature. Plates were washed as before and 100 µl of serum diluted in blocking buffer was added to each well, then incubated overnight at 4°C. Plates were washed four times and horseradish peroxidase (HRP)-conjugated antibodies diluted in blocking buffer was added at 100 µl/well. These included HRP-conjugated rabbit anti-mouse IgG1 (1:4000 dilution), IgG2a (1:2000), IgG2b (1:3000) (Zymed Laboratories, CA), HRP-conjugated goat anti-mouse IgA (1:4000), IgG3 (1:2000), and HRP-conjugated rat anti-mouse IgE (1:2000) antibodies (AbD Serotec). Plates were sealed and incubated for 2 hours at room temperature,

then washed 6 times with wash buffer. Substrate solution (100 µg/ml TMB diluted in DMSO, 0.003% (v/v) H<sub>2</sub>O<sub>2</sub>, in 0.11 M acetate buffer, pH 5.5) was added at 100 µl/well, and the plate was incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µl/well of 1.8 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance values were measured at 450 nm using a FLUOstar optima microplate reader (BMG Labtech, Offenberg).

## **2.25. Transformation of plasmids into *E. coli* SHuffle cells**

*NbVAL3* to *NbVAL8* cDNAs were previously cloned individually into the pET-29b expression vector (Novagen) and verified to be in frame (Huang, 2010). The pET-29b plasmid contains a kanamycin resistance gene and a *lac*-operon (Fig. 2.2). These plasmids were transformed into *Escherichia coli* SHuffle cells for expression testing by heat shock. Competent *E. coli* SHuffle cells (NEB) in 100 µl aliquots were thawed on ice, and 100 ng of each plasmid in 100 µl of transformation buffer (0.1 M KCl, 30 mM CaCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>) was added to the cells and left on ice for 30 minutes. The tubes were immersed in a water bath at 42°C for 30 seconds and immediately transferred back to ice for 5 minutes, after which 1 ml of Luria-Bertani broth (LB, 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% yeast extract, pH 7.0) was added to each tube and placed in a shaking incubator at 37°C for 1 hour. The cells were centrifuged at 16,000 x g for 2 minutes and resuspended in 100 µl LB. This suspension was spread onto pre-warmed LB-agar (LB supplemented with 1.5% (w/v) agar) plates containing 25 µg/ml kanamycin for antibiotic selection, and incubated at 30°C for 16-24 hours. Individual colonies were verified for positivity by colony PCR with primers specific for each variant of *NbVAL* (Table 2.3). Briefly, a small sample of the colony was resuspended in 10 µl of sterile H<sub>2</sub>O, and 5 µl of this was transferred to a PCR tube. The sample was denatured by heating at 95°C for 5 minutes and cooled before the addition of 20 µl of PCR master mix to standard concentrations and reacted under standard cycling conditions (Section 2.9). Amplified products were resolved by agarose gel electrophoresis (1.5%). A band at the correct size indicates that the colony was positive for the corresponding insert.



**Figure 2.2. Vector map of pET-29b.** Coding sequences for signal peptides were removed and cDNAs of *NbVAL3* to *NbVAL8* were individually cloned into pET-29b with a polyHistidine tag at the C-terminal end. Figure is adapted from the Novagen website.

Target	Primer name	Sequence 5' to 3'
<i>NbVAL3</i>	NbVAL3_49-72Nde1	CATATGATCCAGTCGAAATTCAACTGTCCT
	NbVAL3_649-672Not1	GCGGCCGCTATGGTTGCTGGGCCCAATGGTTC
<i>NbVAL4</i>	NbVAL4_58-81Nde1	CATATGGGCGCGTGTCTAAGACGGAGGGC
	NbVAL4_622-645Not1	GCGGCCGCCCTGACAACACACAGACCGTTCTC
<i>NbVAL5</i>	NbVAL5_49-72Nde1	CATATGAACGCCAGATTTGCTCGGCAGATC
	NbVAL5_619-642Xho1	CTCGAGGCTCATATAGCAGAGTGTGCCACC
<i>NbVAL6</i>	NbVAL6_49-72Nde1	CATATGACGCCGATTAGCCCAGTTTCCATG
	NbVAL6_652-675Xho1	CTCGAGTGGGAGTGCCCAATCAGGATAGCA
<i>NbVAL7</i>	NbVAL7_61-84Nde1	CATATGCATGAATACCACTGCAATAAGGAC
	NbVAL7_631-651Xho1	CTCGAGATTATCAGGCGCCTCACAAAGACC
<i>NbVAL8</i>	NbVAL8_91-114Nde1	CATATGACTCAAATTTCAACTGTCAAAC
	NbVAL8_706-729Xho1	CTCGAGGTTTTCTCTTTTCTTAATGCACAA

**Table 2.3. PCR primers for verification of *NbVAL* inserts in pET-29b plasmids.**

## **2.26. Expression testing of recombinant *Nb*VALs in *E. coli* SHuffle cells**

Expression of *Nb*VALs in SHuffle cells were first tested in small-scale cultures. Cells were picked from positive colonies with a sterile loop and inoculated into 5 ml of LB-kanamycin (25 µg/ml). The tubes were incubated overnight in a shaking incubator at 30°C. An aliquot of these overnight cultures was inoculated into a small volume of fresh LB-kanamycin at a 1:100 ratio (10 µl of culture into 10 ml LB), and grown in a shaking incubator at 30°C, 26°C or 16°C until absorbance at 600 nm reaches 0.6 (OD<sub>600</sub> = 0.6). This is taken as the zero-hour time point. At this point the cells were either left in culture without induction, or induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 or 1 mM. The cells were returned to culture at the specified temperatures. At the specified time points, 1 ml of culture was withdrawn into an eppendorf and centrifuged at 16,000 x g for 5 minutes. The cell pellets were lysed in 100 µl of B-PER protein extraction reagent (Pierce) and placed on a rotary mixer for 30 minutes at 4°C, then centrifuged at 16,000 x g for 5 minutes to pellet the insoluble fraction and cell debris. The supernatant containing the soluble fraction was separated from the pellet, which was itself resuspended in 100 µl of the B-PER reagent. Proteins in the soluble and insoluble fractions (10 µl each) were resolved by 15% SDS-PAGE (Section 2.6) and probed with an anti-polyHistidine antibody (1:3000, Sigma) by Western blotting (Section 2.22).

## **2.27. Large-scale expression and purification of *Nb*VALs**

Overnight cultures of the transformed SHuffle cells were grown as described in section 2.26. Each of these were used to inoculate into 1L of LB supplemented with 25 µg/ml kanamycin at a 1:100 ratio. The cultures were grown in a shaking incubator at 26°C for 24 hours after OD<sub>600</sub> has reached 0.6, without IPTG induction. The cells were harvested by centrifugation at 4,000 x g for 30 minutes at 4°C, and the pellets were frozen until further use.

Protein purification was carried out under native conditions at 4°C. The cell pellets were thawed for 15 minutes on ice and resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) at 3 ml per gram wet weight. A protease

inhibitor cocktail (EDTA-free, Sigma) was added to the mixture at 50  $\mu$ l per gram of cells. Lysozyme was added to 1 mg/ml and the tubes were incubated on ice for 30 minutes. The mixture was sonicated on ice using a microtip sonicator with six 30-second bursts at 40 W, with 30-second cooling periods in between. RNase A and DNase I were added to a final concentration of 10  $\mu$ g/ml and 5  $\mu$ g/ml respectively. The tubes were incubated on ice for 15 minutes, then centrifuged at 10,000 x g for 30 minutes at 4°C to pellet the cellular debris. The supernatant was syringe-filtered through a 0.4  $\mu$ m membrane and tested for the presence of the recombinant protein by Western blotting.

Lysates containing the His-tagged recombinant protein was purified by Nickel-chelate affinity chromatography at 4°C (Qiagen). Typically, 1 ml of 50% Ni-NTA slurry equilibrated with lysis buffer was added per 4 ml of cleared lysate and mixed on a rotary mixer for 90 minutes. The lysate-Ni-NTA mixture was loaded onto a column, and the flowthrough was collected into a tube. The resin was washed twice with 10 ml of wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0), and a third time with wash buffer containing 40 mM imidazole. Finally, the recombinant protein was eluted in 0.5 ml fractions with an elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0) and collected separately. Samples of the flowthrough, wash fractions and eluates (10  $\mu$ l each) were analysed by SDS-PAGE. Fractions containing pure recombinant proteins were dialysed against PBS (3 x 2L) in dialysis tubing with a molecular weight cut-off at 7,000 kDa. The final protein concentration was determined by the BCA assay (Section 2.5).

## **2.28. LPS removal**

LPS was removed from protein preparations using the Detoxi-gel endotoxin removing column (Pierce) according to manufacturer's instructions. Briefly, the resin was washed with five resin-bed volumes of 1% sodium deoxycholate, then washed and equilibrated with ten resin bed volumes of sterile buffer (PBS). The column was capped and the sample was applied to the column, where they were incubated with the resin for 1 hour. The samples were eluted with sterile PBS in 0.5 ml fractions.

### **2.29. Immediate hypersensitivity skin test**

The fur of subject mice was removed from the abdomen with depilatory cream (Veet) 2 days before the experiment. Mice were placed in a heat box at 37°C for 10 minutes prior to injection with 100 µl of 0.5% (w/v) Evans blue (Sigma) in PBS through the tail vein. Each mouse was then anaesthetised with 30 µl of 100 mg/ml Ketaset and 15 µl of 20 mg/ml xylazine. Mice were intradermally challenged on the abdomen with 1 µg of *NbVAL4* or *NbVAL7*, 500 ng of mast cell activating compound C48/80 (positive control, Sigma) or PBS (negative control), in a total volume of 15 µl. Extravasation of Evans blue was assessed 15 minutes post-challenge.

### **2.30. Immunisation of mice with *NbVAL7***

Female Balb/c mice were immunised with *NbVAL7* precipitated with alum as adjuvant. Equal volumes of *NbVAL7* (0.5 mg/ml in PBS) and 9% (w/v) aluminium potassium sulphate was mixed in a tube. One drop of phenol red indicator dye was added to the mixture, which turns the solution yellow. The solution was neutralised by the dropwise addition of 1 M NaOH until the indicator turned pink. The solution was left to stand at room temperature for 30 minutes. The tubes were centrifuged at 3000 x *g* for 10 minutes, and the supernatant was withdrawn. A sample of this (25 µl) was resolved by SDS-PAGE to verify that the precipitation was successful. The pellet was washed four times with PBS and resuspended in PBS to a final concentration of 0.25 mg/ml of *NbVAL7*. Each mouse was immunised with 100 µl of this suspension (25 µg of *NbVAL7* per mouse) by subcutaneous injection. Control mice were injected with an equal amount of precipitated alum in parallel. Mice were boosted twice with 15 µg of *NbVAL7*-alum (or just alum for controls), with 4 weeks of resting period in between each immunisation.

### **2.31. Measurement of proliferation and cytokine release by mouse splenocytes**

Spleens were harvested from sacrificed mice and minced finely with sharp forceps in cold sterile RPMI-1640. The pieces were shredded by passing through a 18G needle

syringe and passed through a 100 µm cell strainer. The cells were centrifuged for 5 minutes at 300 x g at 4°C and washed twice with cell culture medium. Red blood cells were lysed by the addition of 0.83% ice-cold ammonium chloride (1 ml per spleen) for 2-3 minutes with gentle agitation. The cells were washed twice and a sample of cells in trypan blue was quantified in a haemocytometer. The cells were seeded at 4 x 10<sup>5</sup> cells/well in a 96-well cell culture plate. Antigen sample (adult secreted products) diluted in cell culture medium was added to the cells to a final protein concentration of 10 µg/ml in a total volume of 200 µl per well. Cells were cultured for 72 hours at 37°C, 5% CO<sub>2</sub>. The culture medium was withdrawn from the wells and used for cytokine analysis. <sup>3</sup>H-thymidine was added to the cells (1 µCi per well) and cultured for another 16 hours. Cells were collected onto a filter disc using an automated 96-well plate cell harvester, after which radioactive counts (cpm) were assessed via a beta-counter to determine the levels of cell proliferation.

Cytokine ELISA analysis was used to detect cytokine levels in cell culture supernatants. The procedures were similar to those of the antigen-specific serum ELISA, but with the following modifications. Maxisorb Surface 96-well plates (Nunc) were coated with 50 µl/well of capture antibody, the range of which included anti-mouse IL-4 (2 µg/ml), anti-mouse IL-5 (0.5 µg/ml), anti-mouse IL-10 (2 µg/ml) and anti-mouse IFN $\gamma$  (1.3 µg/ml), diluted in PBS. The plates were sealed and incubated overnight at 4 °C. The coating solution was then aspirated and the wells washed 3 times with wash buffer (0.05% (v/v) Tween 20 in PBS). The plates were blocked by the addition of 200 µl/well of blocking buffer (1% BSA (v/v) in PBS) and incubated for 2 hours at room temperature, after which the solution was aspirated and the wells were washed 3 times with wash buffer. Test supernatant or serially diluted cytokine standards (in blocking buffer) ranging from 125 pg/µl to 8000 pg/µl were added at 50 µl/well and incubated overnight at 4 °C. The plates were washed 4 times with wash buffer prior to the addition of 50 µl/well of anti-mouse biotinated detection antibody for IL-4 (0.1 µg/ml), IL-5 (0.1 µg/ml), IL-10 (0.5 µg/ml) and IFN $\gamma$  (0.5 µg/ml), diluted in blocking buffer, and incubated for 2 hours at RT. The plates were washed 6 times with wash buffer and 50 µl of avidin-horseradish peroxidase (HRP) diluted 1:1000 in blocking buffer was added per well. The plates were incubated for 30 minutes at RT, then washed 6 times with wash buffer. Substrate solution (100 µg/ml TMB diluted in DMSO, 0.003% (v/v) H<sub>2</sub>O<sub>2</sub>, in 0.11 M acetate buffer pH 5.5) was

quickly added at 100  $\mu\text{l}$ /well and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50  $\mu\text{l}$ /well of 1.8 M  $\text{H}_2\text{SO}_4$ , and the absorbance values were measured at 450 nm using a FLUOstar optima microplate reader (BMG Labtech, Offenberg).

### **2.32. Assay for skin penetration**

The skin of euthanised and shaved rats was excised from the abdomen and cut into sections measuring approximately 4 cm in diameter. Each section was sandwiched between two 20 ml syringe barrels clamped together with bulldog clips, with the lower syringe filled with warm PBS in contact with the underside of the skin. The setup was placed in a 37°C water bath in which the lower chamber was submerged. The integrity of the skin was checked by adding 1 ml of PBS to the upper chamber for 15 minutes. L3 (3000 worms / group in triplicates) were incubated in 50  $\mu\text{l}$  of either PBS, naïve mouse serum, hyperimmune mouse serum, or antisera against VAL proteins produced in mice, for 1 hour at room temperature. An additional triplicate group of L3 worms were heat-killed at 65°C for 10 minutes (negative control). Each group of L3 was then added to the upper chamber of the apparatus to the 1 ml of PBS already present. The L3s were allowed to migrate for 30 minutes in the setup. The remaining L3 were collected from the upper chamber, which was then washed with 4 x 1 ml PBS into the collection tube and counted. The number of migrated L3 was calculated by subtracting the number of remaining L3 from the negative control.

### **2.33. MTT viability assay for adult worms**

Adult worms were isolated and washed extensively with PBS. The worms were aliquoted in triplicate into the wells of a 96-well plate, containing 70 worms in 200  $\mu\text{l}$  PBS. Additional groups of worms were heat-killed at 65°C for 10 minutes in triplicate as a background level control. A 5 mg/ml solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS was filter sterilised and 20  $\mu\text{l}$  of this was added to each well. The plate was put into culture at 37°C and 5%  $\text{CO}_2$  for 6 hours, after which 200  $\mu\text{l}$  of the supernatant was removed from each well and



replaced with 200  $\mu$ l of DMSO to solubilise the dye and the worms, with shaking at 37°C for 2 hours. ‘Blank’ wells were subjected to the same treatment as the test wells throughout the assay but without any worms. Absorbance was measured at 540 nm.

### **2.34. Statistical analysis**

Graphs were plotted using Prism 4 (GraphPad Software) or Microsoft Excel. Error bars represent the standard error of the mean (SEM), and statistics were performed using the unpaired Student’s *t*-test assuming unequal variances, unless otherwise stated. Levels of significance are indicated as follows: Three symbols:  $P < 0.001$ , two symbols:  $P < 0.01$ , one symbol:  $P < 0.05$ . The sample sizes used in the statistical testing were represented by *n* and shown in figure legends where appropriate.

For proportionate data, the uncertainty level was quantified by a 95% confidence level (CI), calculated using the modified Wald method on the GraphPad QuickCalc web calculator (<http://www.graphpad.com/quickcalcs/ConfInterval1.cfm>). In such cases this is clearly stated in the figure legends and represented by the error bars. For ranked data, the level of significance was determined by the Mann-Whitney rank sum test using the Graphpad Prism software.

# **Chapter 3**

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**Comparative studies of laboratory  
passaged and a recent field isolate  
of *N. brasiliensis***

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### 3.1 Introduction

The traits of all living species are shaped by the life history and experience of their ancestors through natural selection and evolution. Nematodes in particular are highly ubiquitous forms of life with complex diversities in nature, which is perhaps an indicator of their adaptability and trait plasticity in the face of selection. For research in helminthology, a subset of work distinctive to other branches of parasitology is the need to propagate the parasite life cycle by serial passages in laboratory animals. In the case of *N. brasiliensis*, passaging infective larvae through new host rats at least once every six weeks is necessary to keep the stock alive. Although this process facilitates research by making parasite stocks readily available, and makes results more replicable from the use of a relatively homogeneous stock, it is a somewhat artificial process. It is not known whether the act of serial laboratory passage itself has effects on the life history traits of the parasite, and whether a stock with these putative changes may misrepresent natural infection. Experimental data collected from the use of parasitic nematodes maintained in the laboratory are often used to address immunological issues and predict epidemiological models, which may contribute to the design of drugs and vaccines for use in the natural setting. If a laboratory-maintained model fails to correctly represent natural infection, these predictions may become inaccurate and possibly compromise drug and vaccine efficacy. Therefore, studying the effects of laboratory adaptation on parasitic nematodes is important, and is the focus of this chapter.

Parasites are a particularly interesting group of organisms in the study of evolution, because the selective forces acting on parasite traits are influenced by their interplay with their hosts, which, through generations, may themselves evolve in response to the parasite, forming a dynamic of co-evolution unlike any other forms of life (Anderson and May, 1982; Hafner and Nadler, 1988). As the lifespan of animal hosts is often longer than that of the parasite, the parasite may cycle through many generations within a single generation of the host, and thus represents the more rapidly evolving party of the two (Anderson and May, 1982). Parasitic nematodes can undergo adaptations according to their life history conditions. Repeated passage of nematodes through specific hosts can have profound influences on their biological traits. For example, many parasitic nematodes can be adapted to infect laboratory

animal species which are not their natural hosts via serial passage. The human hookworm *Necator americanus* can be adapted to infect hamsters (Sen, 1972), and *N. brasiliensis* can be adapted to mice and hamsters (Haley, 1966a, b; Solomon and Haley, 1966; Wescott and Todd, 1966). In contrast, restrictive serial passage of the nematode *Heligmosomoides polygyrus* through ten generations in the Quackenbush mouse increased the parasite's infectivity in this particular mouse strain but not others (Dobson and Owen, 1977). Propagation of *H. polygyrus* through immune mice rather than naïve mice also selected for a strain which performed better in subsequent infections in terms of survival and fecundity, particularly in secondary infections (Su and Dobson, 1997). Similarly, *Trichostrongylus colubriformis* passaged through vaccinated sheep survived better in infections than those propagated through naïve sheep, with the adaptation evident after a single passage (Wendon, 1990). At the other end of the spectrum, repeated passage of a field isolate of *Haemonchus contortus* through immune or susceptible (immunosuppressed) sheep did not result in a difference in infection kinetics, even after fourteen generations of parasite passage (Albers and Burgess, 1988; Woolaston *et al.*, 1992). This shows that there is a degree of variability in the results of these selection experiments between different host-parasite combinations, which may be due to qualitative differences in host-parasite relationships, or differing selection regimes.

Infection outcome is balanced by the dynamic interactions between the parasite and the host, in which life history traits such as the infectivity, establishment, survivorship and fecundity of the parasite are important parameters. As in most other organisms, the life history traits of the parasitic stages of gastrointestinal helminths are subject to density-dependent effects, in which case the parasite's abundance and aggregation in the host regulates and stabilises infection kinetics (Keymer, 1982; Quinnell *et al.*, 1990; Scott and Lewis, 1987). Density is usually constrained by an upper limit of virulence and infectivity, as it is rarely in the interest of the parasite to kill its host (Andre *et al.*, 2003; Keymer and Dobson, 1987; Scott and Lewis, 1987). For GI nematodes, density (i.e. the parasite worm burden in the host gut) generally shows a negative correlation with fecundity and/or survivorship, without significant effects on initial worm establishment (Paterson and Viney, 2002; Quinnell *et al.*, 1990; Stear *et al.*, 1999). In some cases decreased fecundity can be partially attributed to a decrease in survivorship, whereas in other cases it can be completely independent (Keymer and

Slater, 1987; Stear *et al.*, 1999). The basis of these density-dependent effects has been debatable, as it was not clear whether within-host intraspecific competition for resources such as nutrients or space is responsible, or if they were brought about by the effects of the host immune response. A higher worm density is likely to escalate the severity of host defence mechanisms, which in turn may possibly reduce worm survivorship and/or fecundity. It has later been demonstrated that survivorship and fecundity are not constrained by density-dependent effects in immuno-compromised nude mice however, suggesting that host responses can act to regulate the infection before constraints from intraspecific competition in the host gut could take place (Paterson and Viney, 2002). Since the evolution of parasitic nematodes are largely confined by the conspecifics within a host and the immune response, it has been proposed that life-history traits such as survivorship and fecundity may trade-off against other traits which would be favourable for its propagation during selection (Paterson and Barber, 2007).

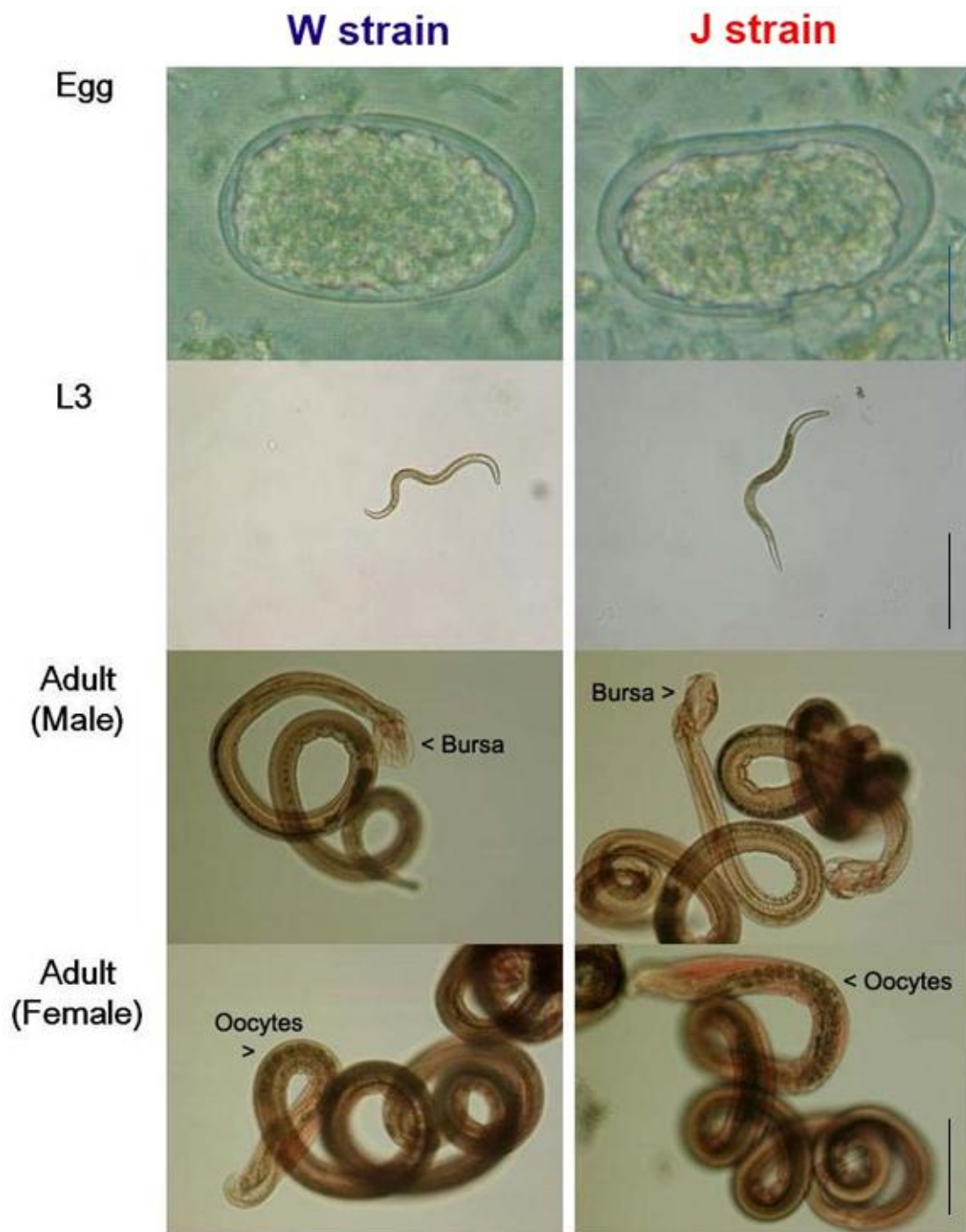
Much of what is currently known of the biology, host-parasite relationship and immunology of *N. brasiliensis* infections came from experiments utilising a strain which originated from the Wellcome laboratories in 1958 (Jenkins, 1972). In recent years it has been suspected that this Wellcome (W) strain is displaying altered infection dynamics including reduced egg output. However, these observations have not been documented and quantified. At the start of this project, a strain of *N. brasiliensis* recently isolated from a wild rat in Japan was donated to our laboratory. As this Japanese field strain (J) is representative of the parasite in its natural environment, this provided a window of opportunity to define the infection dynamics and compare it with the laboratory passaged W strain.

A comparison of the ribosomal RNA gene (rDNA) sequences was first carried out between the W and the J strain to confirm the latter's identity as *N. brasiliensis*. The infection dynamics of the two strains were then compared, as well as the host response to infection. The secreted protein profiles of the two strains were also compared. Lastly, the activity of two known nematode secreted enzymes, acetylcholinesterases and nucleotide metabolising enzymes, were determined.

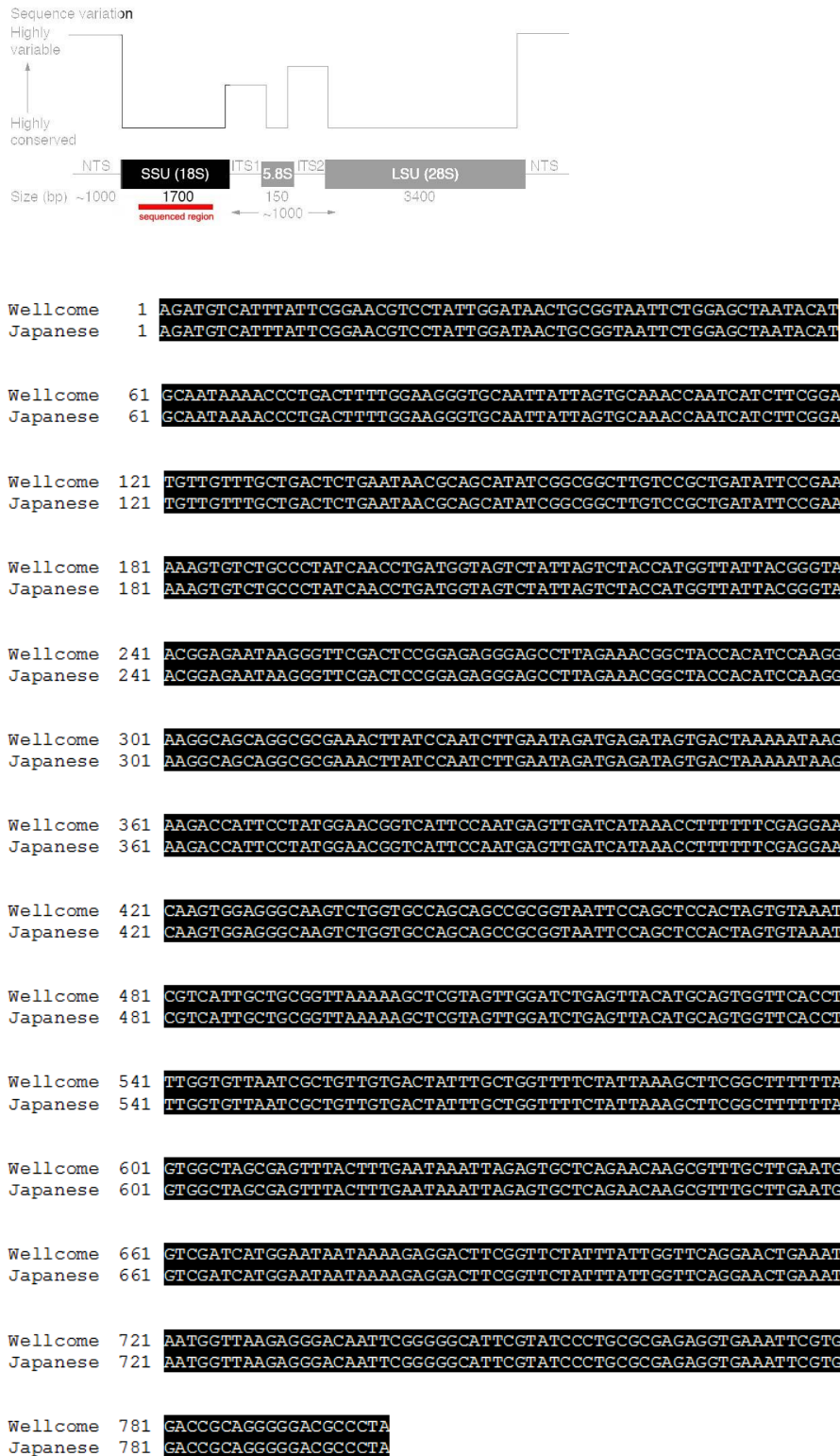
### 3.2. Confirmation of identity of the Japanese strain

Eggs, L3 and adult worms of the W and J strain were observed to be morphologically indistinguishable by light microscopy (Fig. 3.1). The anatomical features of *N. brasiliensis* are most distinctive at the adult stage. Freshly collected adult worms were bright red in colour from the intrinsic globin produced by the worms, and the females were larger and more intensely red than the males. At the posterior end, the male was distinguished by a copulatory bursa, whereas the females had a curved, conical end with a cuticle covering the vulva and the anus, with developing oocytes clearly visible in the ovary. The eggs produced by the two strains were similar in size and shape, with an egg shell protecting the developing cells. The L3 of both strains appeared identical, moved in the same manner, and both were attracted to warmth and/or light. Based on morphological criteria, the two strains thus appeared to belong to the same species.

To confirm that the Japanese isolate could be classified as *N. brasiliensis*, a comparison of sequences within the ribosomal RNA gene (rDNA) cistron was carried out between the W and J strain. Within this cistron there are regions of sequence variability which are frequently used as phylogenetic markers in nematodes and other organisms (Blaxter *et al.*, 1998; Dorris *et al.*, 1999). Three regions of the rDNA cistron were chosen for analysis: the small subunit (SSU) gene, the internal transcribed spacers (ITS)-1 and ITS-2, in ascending order of sequence variability (Fig. 3.2, top panel). Nucleotide sequences within these regions were obtained by PCR amplification of genomic DNA, product purification and sequencing. Alignments of the SSU, ITS-1 and ITS-2 sequences from both strains are shown in Fig. 3.2, 3.3 and 3.4 respectively. The sequences of SSU, ITS-1 and ITS-2 were all 100% identical between strains. Since the SSU region consists of a coding, structural sequence of a functional gene, it was perhaps not surprising that this region was completely conserved. The ITS-1 and ITS-2 regions are highly variable between different nematode species, and their invariability between the strains suggests that the W and J strain are extremely phylogenetically conserved (Fig. 3.3 and 3.4).

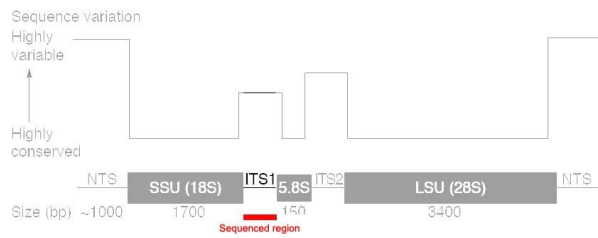


**Figure 3.1.** The Wellcome strain (left panel) and the Japanese strain (right panel) of *N. brasiliensis* are anatomically indistinguishable for the egg, L3 and adult stages. Eggs were photographed at x1600 magnification, L3 and adults at x100 magnification. The copulatory bursa of the male adults and the oocytes within the ovary of the female adults are indicated with arrows in the diagram. Scale bar (blue): 25  $\mu\text{m}$ . Scale bar (black): 400  $\mu\text{m}$ .



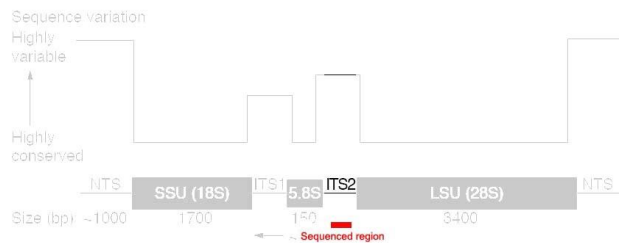
**Figure 3.2. Alignment of the rDNA small subunit gene (SSU) sequences between the W and J strain of *N. brasiliensis*.** SSU sequences from the W and J strains were aligned using Multialign and presented using Boxshade software. The location of this sequence within the rDNA cistron is shown at the top. Identical nucleotides are shaded in black.





Wellcome	1	GGTTAATTCGCGTGATCTGAGAACCCGACGCGCTTGTCGTTACGGCTTTGCCGTAGCGGT
Japanese	1	GGTTAATTCGCGTGATCTGAGAACCCGACGCGCTTGTCGTTACGGCTTTGCCGTAGCGGT
Wellcome	61	CGGTAGTATCATACCAACCGTTAGAGCTCTACATTTAGAGGTGTCTACGTGTATGGATGA
Japanese	61	CGGTAGTATCATACCAACCGTTAGAGCTCTACATTTAGAGGTGTCTACGTGTATGGATGA
Wellcome	121	ATCGTTCTTGAGTGACGGTGATGATTTTCGTACGCAAAGTTCCCATTCGTGGTTGAGCTTC
Japanese	121	ATCGTTCTTGAGTGACGGTGATGATTTTCGTACGCAAAGTTCCCATTCGTGGTTGAGCTTC
Wellcome	181	TAGACTTAATGAGCATTGCGTGAATGCCACCTCTTAAACATATATTTTCAATTGTATTGA
Japanese	181	TAGACTTAATGAGCATTGCGTGAATGCCACCTCTTAAACATATATTTTCAATTGTATTGA
Wellcome	241	TTATATTTGGTGATTAGGTGTGTATGTGTTATTATACATTCGTGTATAATGCGCGTACAT
Japanese	241	TTATATTTGGTGATTAGGTGTGTATGTGTTATTATACATTCGTGTATAATGCGCGTACAT
Wellcome	301	TCCATACCTGGTTCGTTTCAGGAAATCTTAATGATCCGCCTCTGAGTGGACGCCAAAACAC
Japanese	301	TCCATACCTGGTTCGTTTCAGGAAATCTTAATGATCCGCCTCTGAGTGGACGCCAAAACAC
Wellcome	361	ACTTTTTACTGTATGTTGCTTGCAGAATTTAGTGTTATTTTCGAATAAACACTATACAC
Japanese	361	ACTTTTTACTGTATGTTGCTTGCAGAATTTAGTGTTATTTTCGAATAAACACTATACAC
Wellcome	421	TT
Japanese	421	TT

**Figure 3.3. Alignment of the rDNA internal transcribed spacer 1 (ITS-1) sequences.** ITS-1 sequences from the W and J strains were aligned using Multialign and presented using Boxshade software. The location of this sequence within the rDNA cistron is shown at the top. Identical nucleotides are shaded in black.



Wellcome	1	GCAGAATAATATCATACTGCATGTTACATGTGTGTATGATATTGCTGAATGAAATCGATG
Japanese	1	GCAGAATAATATCATACTGCATGTTACATGTGTGTATGATATTGCTGAATGAAATCGATG
Wellcome	61	AACATTATAGACGTTTATCACACTATTATGATAAGTACTTGTGAATGAGAAGGAACATCA
Japanese	61	AACATTATAGACGTTTATCACACTATTATGATAAGTACTTGTGAATGAGAAGGAACATCA
Wellcome	121	TAATGGTACCGCTTGTGTTGTG
Japanese	121	TAATGGTACCGCTTGTGTTGTG

**Figure 3.4. Alignment of the rDNA internal transcribed spacer 2 (ITS-2) sequences.** ITS-2 sequences from the W and J strains were aligned using Multialign and presented using Boxshade software. The location of this sequence within the rDNA cistron is shown at the top. Identical nucleotides are shaded in black.

The ITS-1 and ITS-2 sequences of seven other members of the nematode order Strongylida – *Carolinensis minutus*, *Ohbayashinema erbaevae*, *Heligmosomoides polygyrus*, *Nematodirus battus*, *Nematodirus filicollis*, *Haemonchus contortus* and *Teladorsagia circumcincta* - were retrieved from Genbank for comparison with the sequences of the W and J strain of *N. brasiliensis*. The alignments for ITS-1 and ITS-2 are shown in Fig. 3.5 and Fig. 3.6 respectively, with both regions displaying considerable sequence variability between the different species. The alignment matrices for ITS-1 and ITS-2 were concatenated within the Mesquite system for phylogenetic computing (Maddison and Maddison, 2010) and used to construct a phylogenetic tree on Phylogeny.fr (Dereeper *et al.*, 2008), utilising algorithms based on the neighbour-joining (NJ) method to determine phylogenetic distances, then verified using maximum likelihood (ML) analysis (Fig. 3.7). Bootstrap values were annotated, with all branches but one supported by a value of 1, showing that the tree is extremely well-supported. Branch lengths are indicative of how closely related the species are to each other, proportional to the amount of inferred evolutionary changes. W and J strains of *N. brasiliensis* clustered together. Of all the other species, *C. minutus* was determined to be the most closely related to *N. brasiliensis*, in accordance with the published literature (Audebert *et al.*, 2005). They were also shown to have a common root with *O. erbaevae* and *H. polygyrus*, which is reflective of their phylogenetic relationship in that all four species are members of the Heligmosomoidea family within the order Strongylida. Similarly, *N. battus* and *N. filicollis*, both members of the Molineoidea family, are sub-grouped together. *H. contortus* and *T. circumcincta*, two closely related nematodes of small ruminants belonging to the Trichostrongylidae family, form a sub-group (Audebert *et al.*, 2005). These analyses show that ITS-1 and ITS-2 are good phylogenetic markers for distinguishing between closely related nematode species, as well as the categorisation of families within an order. Therefore, these data validate the use of ITS-1 and ITS-2 for species identification, and strongly confirm the identity of the J isolate as a strain of *N. brasiliensis*.

W *N. brasiliensis* 1 GGTTA...A.TTCG.CGTGATC.TGAGAACCCGACCGCT...T..GTCGTTACGGC.TTT  
 J *N. brasiliensis* 1 GGTTA...A.TTCG.CGTGATC.TGAGAACCCGACCGCT...T..GTCGTTACGGC.TTT  
*C. minutus* 1 GGTCC...CGATCGACGTGATCGTATAACCCGACTTGCTAGT..GTCGTTACGGCCATA  
*O. erbaevae* 1 GGTTCTTCGTTAGATGTATTC..GAGAAACCAACTGCTAGTATGTTGTACGACCA.C  
*H. polygyrus* 1 GGTTCTTCGTTAGATGTATTC..GAGAAACCAACTGCTAGTATGTTGTACGACCA.C  
*N. battus* 1 GGTTCC...GTTTGTATCGCCTC..GAGAAACCAACCGACTGGC..ATGTTTTCGCGCCA.T  
*N. filicollis* 1 GGTTCC...GTTTGTATCGTAC..GAGAAACCAACCGACTGGM..ATGTTTTCGCGACAT.T  
*H. contortus* 1 GGTTCC...CTTTGATC...AC..GAGAAACCAACTGCTA...T..GTTTTCGACTT.T  
*T. circumcinota* 1 GGTTCC...CTTTGATC...AC..GAGAAACCAACTGCTT...C..ATTTTACGACTT.T

W *N. brasiliensis* 50 CCCGTAGCGGTTCGGTAGT..ATCATACCA...ACCGTTAGAGCTCTACATTTA.GAGGTGT  
 J *N. brasiliensis* 50 CCCGTAGCGGTTCGGTAGT..ATCATACCA...ACCGTTAGAGCTCTACATTTA.GAGGTGT  
*C. minutus* 56 GTCGTAAACGGTTCGGTTGTTATCAACCATTACCCTTAGAGCTCTAAACTATGAGGTGT  
*O. erbaevae* 58 GTCGT..CAAACGTTGGTAGTATCGACC...ACCGTTAGAGCTC...ACATGTAAGGTGC  
*H. polygyrus* 59 GTCGT..CAAACGTTGGTAGTATCGACC...ACCGTTAGAGCTC...ACATGTAAGGTGC  
*N. battus* 52 GTCGC...GAACGTTGGGAGTATCACCC...CC.GTTGGAGCTCTA.T...GGAGGTGT  
*N. filicollis* 52 GTCGT...AAACGTTGGGAGTATCACCC...CCCGTTGGAGCTCTA.T...GTGAGGTGT  
*H. contortus* 46 GTCGT...AAAGTTGGGAGTATCACCC...CC.GTTAAAGCTCTA.TTACATGAGGTGT  
*T. circumcinota* 47 GTCGT...GAAAGTTGGGAGTATCACCC...CC.GTTAGAGCTCTA.T.ATGTGAGGTGT

W *N. brasiliensis* 105 CTACGTGTATGGATGAATCGTTCTT..GAGTGAACGGTGATGATT...TCGTACGCAAAGTT  
 J *N. brasiliensis* 105 CTACGTGTATGGATGAATCGTTCTT..GAGTGAACGGTGATGATT...TCGTACGCAAAGTT  
*C. minutus* 116 CTACGTGTACGGATGAGTCGTTTAAAGAGTCCGGACTGTGATT...CTGCGCAAAGTT  
*O. erbaevae* 110 CTATGTATGTCGATGAGTCGTTTGTGAGTGAACGGCAGTGATTCTGACATAACGGCGGTA  
*H. polygyrus* 111 CTAGGTATGTCGATGAGTCGTTTGTGAGTGAACGGCAGTGATTCTGACATAACGGCGGTA  
*N. battus* 101 CTACCGTACGGATGAGTCGTTCTT..GAGTGGCGGCAGTGATCG.TCCT..GCAAAGTT  
*N. filicollis* 102 CTACCGTACGGATGAGTCGTTCTT..GAGTGGCGGCAGTGATTCTG.TCCT..AAAGTT  
*H. contortus* 98 CTAT.GTATGACATGAGCCGTTCCA..GAGTGGCGGCAGTGATTGT.TCAT..GCAAAGTT  
*T. circumcinota* 98 CTAT.GTATGACATGAGTCGTTCTT..GAGTGGCGGCAGTGATTGT.TCAT..GCAAAGTT

W *N. brasiliensis* 161 CC.....TTCGTGG.....TTGAGCTTC  
 J *N. brasiliensis* 161 CC.....TTCGTGG.....TTGAGCTTC  
*C. minutus* 172 CC.....CACTGTAGGTGG.....TTGAGCTTC  
*O. erbaevae* 170 ACGTA.....CGTCAACGTA.....CGGTGCGTTGTGTCGGGTC...TTCGCGGCTC  
*H. polygyrus* 171 CCGTAACCGACGCGTAACGGCGGGGGTGGTACGTTGTGTCGRTCGCGCATGCGGTT  
*N. battus* 157 CCCAC.....CTA.....CGGTGG.....TCGAGCTTA  
*N. filicollis* 158 CCCAC.....ATA.....AGGTGG.....TCGAGCTTG  
*H. contortus* 153 CCTAT.....CAT.....TGATGG.....TTGAGCTT  
*T. circumcinota* 153 CCTAT.....CACCTTTATTGGTGTGG.....TTGAGCTT

W *N. brasiliensis* 181 TA....GACTTAATGAGCATTGCGTGAATGCCA CCTCTTACATATATTT.TCAATTGT  
 J *N. brasiliensis* 181 TA....GACTTAATGAGCATTGCGTGAATGCCA CCTCTTACATATATTT.TCAATTGT  
*C. minutus* 196 AA....GACTTGATGAGCATTGCTAGAATGCCGCTCA.ACCCATATTTAT.T.TTTGT  
*O. erbaevae* 215 GATG.AGCCTTGATGAGCATTGCTACAATGCCGCTTACACAAACGTTGTTGTGCAAC  
*H. polygyrus* 231 GACATAGGCTTGATGAGCATTGCTACAATGCCGCTTACACAAACGTTGTTGT.TCAAT  
*N. battus* 180 TT...AGACTTAATGAGTATTGCTGGAATGCCGCTTACTGT.....TTTAC  
*N. filicollis* 181 CT...AGAYTTGATGAGCATTGCTGGAATGCCGCTTACTGT.....TTTAC  
*H. contortus* 175 .G...AGACTTAATAAGTATTGCTATAAATCTGCCTCACCGT.....TTTAT  
*T. circumcinota* 184 .T...AGACTTAATGAGTATTGCTATAAATCTGCCTCATAGT.....TTTAT

W *N. brasiliensis* 236 ATTGATTATATTTG.GTGATTAGGTGTGTATCTGTTATTATACATTCGTTATATGCGC  
 J *N. brasiliensis* 236 ATTGATTATATTTG.GTGATTAGGTGTGTATCTGTTATTATACATTCGTTATATGCGC  
*C. minutus* 248 AGAGAA...AGTTG.GTGATTAGGTGGTTCTGTG.....CTTCGTTCCACAC.CAC  
*O. erbaevae* 274 GTTGATTG...CGCGTTGTTGGTGGTTAGA.....CTTGGTCACTGATGACT  
*H. polygyrus* 289 GTTGATTATACAGCCGTTGTTGGTGGTTAG.....CTCCGTGAGCGATGGCA  
*N. battus* 224 GTTG.....GTGACTAGGCAAT.....GCTAGTACCGTGT  
*N. filicollis* 225 GTTG.....GTGATTGAGCACAA.....GCERCCAKGTGC  
*H. contortus* 218 AATG.....GTGTTAAGTACCGAACAAATTACTTCTGAAATATGTGTGTAC  
*T. circumcinota* 227 AATG.....GTGTTAAGTACATACC.....GCTAGGTGTGTAC

Continued next page with figure legend

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W N. brasiliensis 295 GTACATTCCATACCTGGTTCGTTTCAGGAAATCTTAATGATCCG.CCTCTG....A..GTG
J N. brasiliensis 295 GTACATTCCATACCTGGTTCGTTTCAGGAAATCTTAATGATCCG.CCTCTG....A..GTG
C. minutus 294 ATCTATT..ATACCTGGAT..GTTCAGGGAATCTTAATGATCCG..CAGCTAGTCAA..GTG
O. erbaevae 319 GAGTG....AAACCTGGT..GATCAGGAAATCTTAATGATCCGATAGCTGGTTAACATCG
H. polygyrus 338 GAGTG....AAACCTGGT..GTTCAGGAAATCTTAATGATCCGACGGCTGGTTAATGTGCG
N. battus 256 G.....GAACCTGGCTGT..YAGGTAGTCTTAATGATCCGACACAC.....TTG
N. filicollis 257 G.....TAACCTGGCTGT..CAGGAATCTTAATGATCCGACACAC.....CCG
H. contortus 267 T.....GTACCTGGTATATATCAGGAAACCTTAATGATC....ACGC.....GTG
T. circumcinota 261 G.....GTACCTGGTGT..CAGGAAACCTTAATGATC....TCGC.....CTA

W N. brasiliensis 348 GACGCCAAACACAC.....CTTTTACTGTATGTTGCTTGCAGAATTTAGTGTTTATT
J N. brasiliensis 348 GACGCCAAACACAC.....CTTTTACTGTATGTTGCTTGCAGAATTTAGTGTTTATT
C. minutus 348 GACGCCAATTAATCAAAAACACCAATTTATGTGTATAGATTGCAGATTAT.GTGATT.TT
O. erbaevae 373 GACGCCAACG..ACGTGT...GTCAACACGACACACACACACTTTCTATGTTTAAAT
H. polygyrus 392 GACGCCAACG..CGTGTGTTGTCAACACGACACACACACTTTCTATGTTTAAAT
N. battus 298 GCGCCAAAGTAAAGACTCACTTTTTCATTTGACCTTTGCAGAAT..AGTGACT...
N. filicollis 299 GCGCCAAACGTAAAACTAACAACTTTTCTAATTTCAATTTGCACAGAC..CGTGATC...
H. contortus 307 GACGCCATTATAAAAC..ACAAACATTCATTTTACAGTTTGCAGAATTTAGTGTTCACA
T. circumcinota 299 GACGCCATTATAAAAC..ACAAACCTTCTATGTTTAAATTTGCAGAAA..CGTGTTCACA

W N. brasiliensis 401 TTCGAATAAACACTATACACTT
J N. brasiliensis 401 TTCGAATAAACACTATACACTT
C. minutus 406 TTCTTGAAAAAATCACTCACTT
O. erbaevae 428 GTTTGCAGAACCTAATCACTT
H. polygyrus 449 GTTTGCAGAACCTAATCACTT
N. battus 353 ...TTCATGTCACAATCATGA
N. filicollis 354 ...GATGTATCACTAT..AGGA
H. contortus 365 TTCATTTGTGTCACAAATATCG
T. circumcinota 355 TT..TTTGTGTCACAATATATCG

```

**Figure 3.5.** Alignment of ITS-1 sequences from the W and J strain of *N. brasiliensis* and other nematodes from the order Strongylida. The ITS-1 sequences of *C. minutus*, *O. erbaevae*, *H. polygyrus*, *T. circumcinota*, *N. battus*, *N. filicollis* and *H. contortus* were retrieved from Genbank and aligned with the *N. brasiliensis* sequences using Multialign and Boxshade. Identical nucleotides (for 5 sequences or more) are shaded in black.

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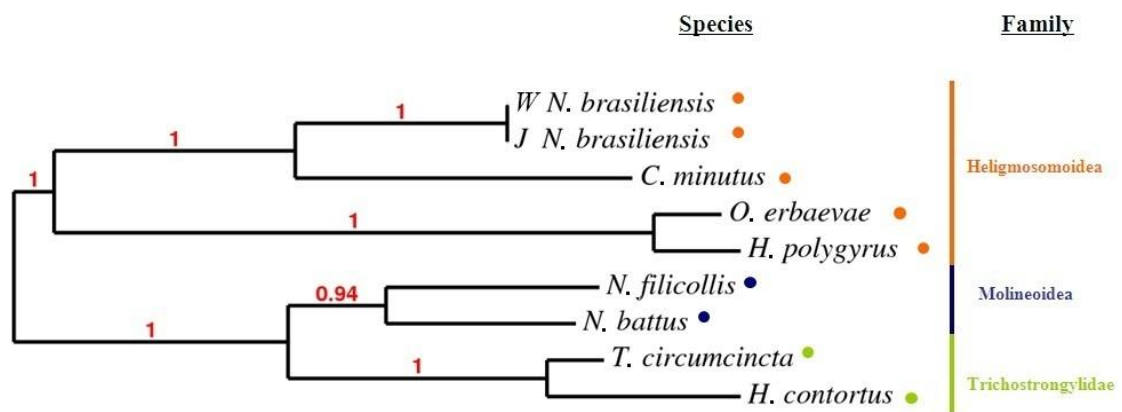
W N. brasiliensis 1 GAATPATAATCCTACTGCATGTTACATGTTGTGTATGATATTCTGAATGAAATC.GATGAA
J N. brasiliensis 1 GAATPATAATCATACTGCATGTTACATGTTGTGTATGATATTCTGAATGAAATC.GATGAA
C. minutus 1 GAATCATAAGTA.GCAACATGGTA.TTGTGAAATACAATATTCTGAATGATCTTGTGAA
O. erbaevae 1 GCAACATAGTGT....CGTCGTCGTCGA...CCACGTCACACTGAATGATGTG.AACACG
H. polygyrus 1 GCAACATAGTGT....CGTCGACGTCGT...CGTCGTCACCTGAATGATGTG.AACACA
N. battus 1 GCAACATGGCTT....CGT.GCTGGTGT...CAAAG..CCCTGAATGATGTG.GACGCG
N. filicollis 1 GCAACGTTGGCTT....TGT.GATGGTGT...CAAAG..CTCCTGAATGATGTG.AACGCG
H. contortus 1 GCAACGTTGATGTT...ATGAAATT.....GTAACATCCTGAATGATATG.AACATG
T. circumcinota 1 GCAACATGACGTACGACGGTATTACCCT...CGTAACGTTCTGAATGATATG.AACGCG

W N. brasiliensis 60 CAT...TATAGACGTTTATCACACTATTATGATAAGTACTTG.TGAATGAGA.AGGAACA
J N. brasiliensis 60 CAT...TATAGACGTTTATCACACTATTATGATAAGTACTTG.TGAATGAGA.AGGAACA
C. minutus 59 CGTATGTATGTGGTGTATCACCCTGGTATGATAAGTACTTGATGAATAAGTGAGGAAAA
O. erbaevae 53 .....ATGTGTGTAGTGTACCGTTTCCTCTA..GTACTCGGCGAGATT.ACATTGAGG
H. polygyrus 53 TTGTGTATGTGTGTAGTGTACCGTTTCCTCTA..GTACTCGGCGAGATT.ACATTGAGG
N. battus 50 .....ATTGTAGCCGTATTGAGTT..CTACTCGATGAGAAAT.GAGATG.AA
N. filicollis 50 .....ATTGTACCGTATCAAAAT..GTCTCGATGAGTAT.GAGATGWT
H. contortus 49 .....T.TGCCAC..TATTTGAGT..GTACTCAGCGAATATTGAGATTGAC
T. circumcinota 57 .....TATTGCTAC..TATTTGAAT..GTACTCAATGAATAT.GAGATTGAT

W N. brasiliensis 115 TCATA..ATGGTACCGCTTGTTTTGTGTGTGTACCCCTTGTTTGTG
J N. brasiliensis 115 TCATA..ATGGTACCGCTTGTTTTGTAATGTTACCCCTTGTTTGTG
C. minutus 119 TCATAATGGTACCGCTTGTTTTGTAATGATAACACACCCGCTAGTCAAC
O. erbaevae 104 ATCGATGAA.ACAGGGCCGGTGTGACACTACAGTACA.CGCCTTC
H. polygyrus 110 ATCGATGAA.ACAGGGCCGGTACGACACTACGACACAGTACATGC
N. battus 92 TTCACCGGGGACTGACTGTAGCCAAATCGATTGTCTCTG.ACATCA
N. filicollis 93 TTGATCGGGGACCTG.CTCTTAACCAATCGATTGTTCA.G.ACATCA
H. contortus 90 TTAGATAGTGACATGTATGGCGACG.ATGTTCTTT.TA.TCATT
T. circumcinota 99 TCASATAGAGACATGTATGGTAATATATCTTCAATGTA.TCATT

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**Figure 3.6. Alignment of ITS-2 sequences between the W and J strains of *N. brasiliensis* and other nematodes from the order Strongylida.** The ITS-2 sequences of *H. polygyrus*, *T. circumcinota*, *N. battus*, *N. filicollis* and *H. contortus* were retrieved from Genbank and aligned with the *N. brasiliensis* sequences using Multialign and Boxshade. Identical nucleotides (for 5 sequences or more) are shaded in black.



**Figure 3.7. Phylogenetic tree of species within the order Strongylida based on the alignment of their ITS-1 and ITS-2 sequences.** The tree was constructed on Phylogeny.fr, utilising algorithms based on the neighbour-joining (NJ) method and confirmed using maximum likelihood (ML) analysis. Nodal support was estimated with the bootstrap procedure using 100 replicates, annotated in red. A bootstrap value of 1 indicates that the branch is completely supported, and values over 0.7 are generally considered to be valid.

### 3.3. Comparative infection dynamics of the *N. brasiliensis* strains

Several parameters which define infection dynamics in a specific host-parasite combination are the parasite's life history traits such as infectivity, establishment, fecundity and survivorship in the host environment, and these were assessed for the W and J strain. Infectivity was measured by the worm burden in infected animals at an early stage of infection, establishment as the number of worms which are present in the host gut as mature adults, fecundity as the number of eggs produced by a set number of adults, and survivorship as the number of adults remaining in the host gut at a defined time late in infection.

Rats were infected with either the W strain or the J strain of *N. brasiliensis* at a dose of 2,000 L3, and sacrificed on days 3, 4, 7, 10 or 13 p.i., when adult worms were recovered from their intestines and quantified (Fig. 3.8). On day 3 and day 4 p.i., many of the worms have arrived at the gut as L4 or young adults. Worm recovery was indistinguishable between strains on both days, indicating no difference in terms of initial infectivity. By day 7 p.i., adult worms were fully mature and established in the gut, and their numbers had peaked and stabilised. Worm recovery on D7 p.i. showed no significant difference between the two strains. The percentage recovery of adults relative to the initial infection dose was  $42.3 \pm 2.7$  % for the W strain, and  $34.8 \pm 8.3$  % for the J strain, suggesting that the two strains are equally capable in terms of establishment in the host. However, a dramatic difference in adult numbers between the strains was seen on day 10 p.i., when the W strain was almost completely expelled by the host, and J strain adults continued to remain in high numbers. Adults of the J strain were eventually cleared by day 13. These results show that the J strain can survive and persist for longer in the host compared to the W strain.

The total egg output resulting from infection was measured by faecal egg count carried out daily from day 3 to day 14 p.i. (Fig. 3.9). There appeared to be a difference in egg output between the two strains. The most obvious difference was that egg production of the W strain was terminated a lot earlier than the J strain (day 8 compared to day 12 p.i.). This is most likely due to that fact that the W strain adults were expelled a lot earlier from the gut, resulting in a much shorter period of egg production. The peak days of egg production were on day 6 p.i. for the W strain, and

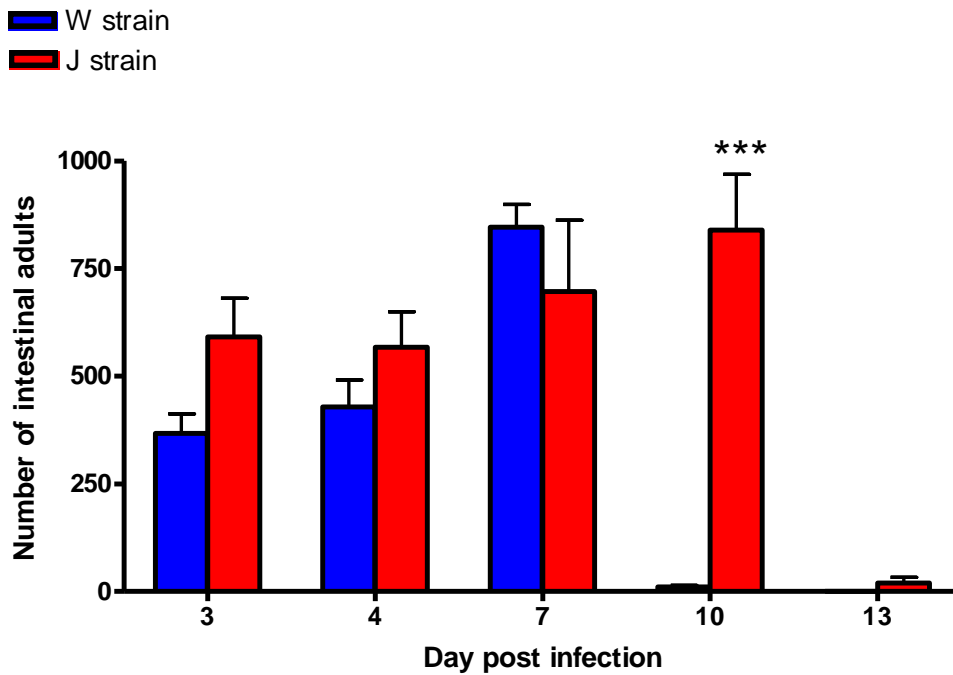


day 7-10 p.i. for the J strain, resulting in a much higher total egg output from the latter. To show this difference quantitatively, the area under each graph in Fig. 3.9 was calculated for an estimate of the total egg output of each strain. Over the course of a primary infection, a total number of 19,085x (where x is the amount of faeces produced by a rat per day in grams) eggs were produced by the J strain, whereas only 5252x eggs were produced by the W strain. This indicates that the total reproductive output of the J strain is over 3.6 times that of the W strain. There was also the interesting observation that egg production started and peaked earlier for the W strain compared to the J strain, although results did not reach statistical significance.

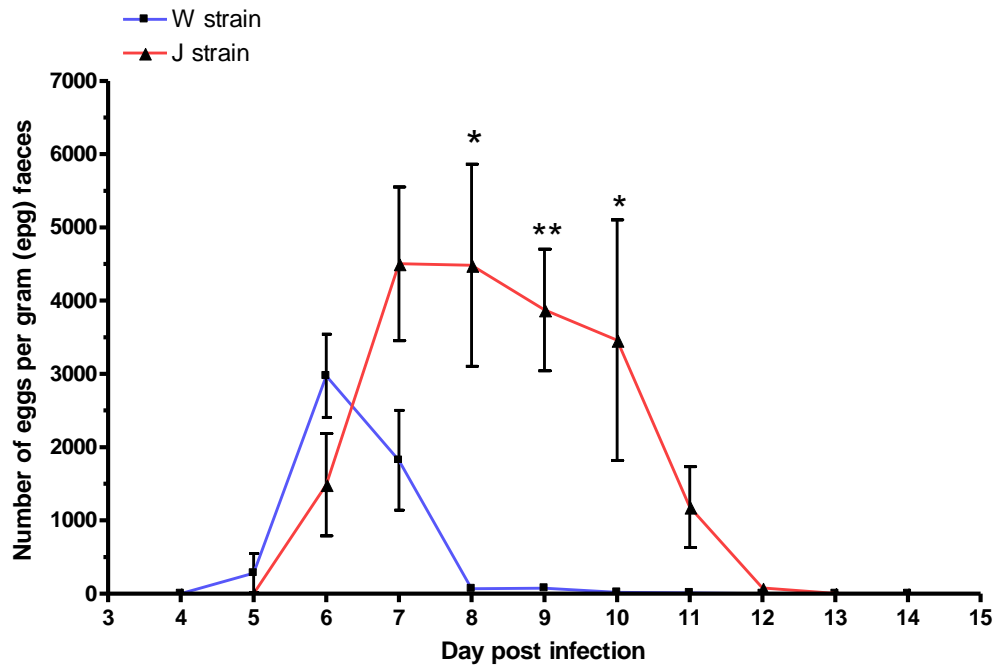
To assess the *per capita* fecundity of the two strains, the number of eggs isolated from faeces was divided by the number of worms present in the host gut on day 7 p.i., when the worms had reached maturity and numbers were stabilised. It was found that the *per capita* fecundity of the W strain was 2.15x per worm, and that of the J strain was 6.47x per worm, i.e. over 3 times that of the former (Fig. 3.10A). *In vitro* egg counts were also carried out to assess the fecundity of the two strains under more controlled conditions, in which the host environment had a lesser role. Fully mature adult worms of the W and J strain were isolated from infected rats on day 7 p.i. and cultured for 24 hours, after which the eggs produced were counted. It was found that the number of eggs produced by the J strain was over 2 times that of the W strain (Fig. 3.10B), indicative of higher fecundity.

The rats were then re-infected six weeks after the primary infection. Re-infection with either strain did not result in any egg production, as determined by faecal egg counts from day 3 to day 7 p.i.. No adult worms were recovered on day 7 p.i., showing that the worms were cleared before maturity. This indicates that protective immunity was elicited in the rats by a primary infection with both strains, mounting an adaptive immune response which eliminated the parasite at an early stage of infection.

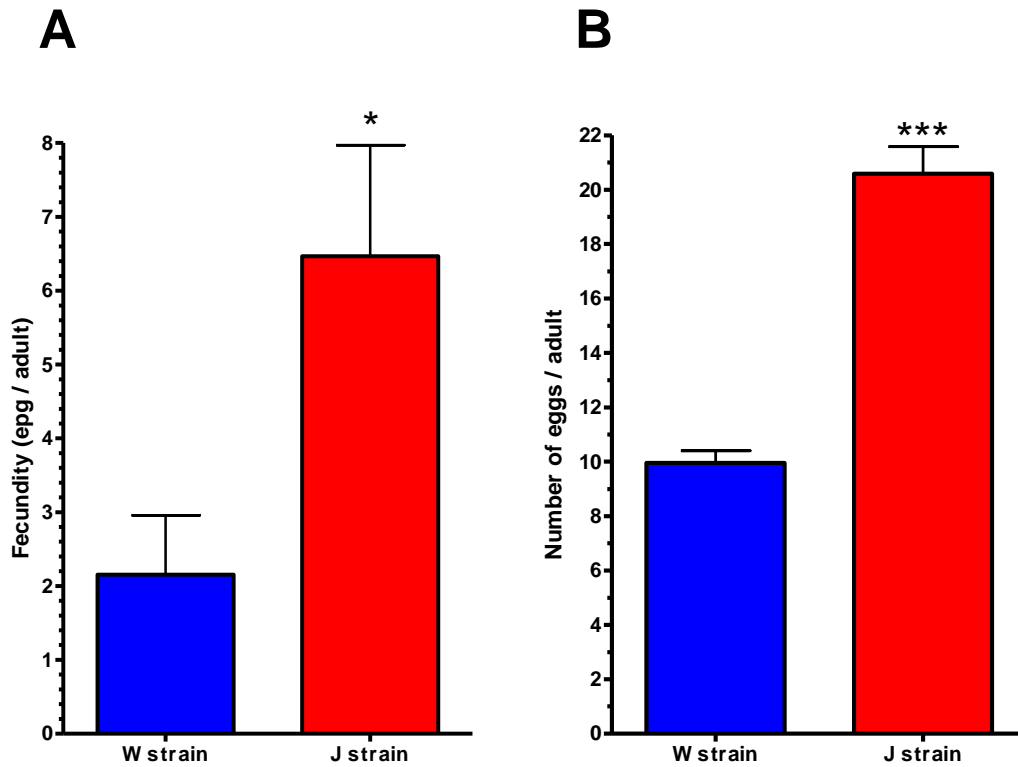
To summarise, the W and J strain of *N. brasiliensis* did not differ in infectivity and host establishment, but both survivorship and fecundity of the J strain were higher. It was unclear to what extent these represented intrinsic differences in properties of the parasite or susceptibility to host immunity, and therefore, the host immune response was also investigated.



**Figure 3.8. Adult worm recovery post-infection.** Rats were infected with 2,000 L3 with either the W strain (blue bars) or the J strain (red bars), and adults were recovered from the intestines at the indicated days post-infection. Results are presented as the mean number of worms recovered + 1 SEM, from 5 rats per group. Asterisks show a significant difference between the strains where \*\*\*  $P < 0.001$  ( $n = 5$ ), determined by the Student's  $t$ -test.



**Figure 3.9. Egg output during infection.** Graphs represent the mean number of eggs recovered per gram of faeces (epg) from rats infected with the W strain (blue) or the J strain (red) at a dose of 2,000 L3. Counts were performed daily from day 3 to day 14 post-infection. Error bars indicate  $\pm 1$  SEM ( $n = 5$  rats per group). Asterisks indicate a significant difference between the strains on a given day, \*\*  $P < 0.01$ , \*  $P < 0.05$ .



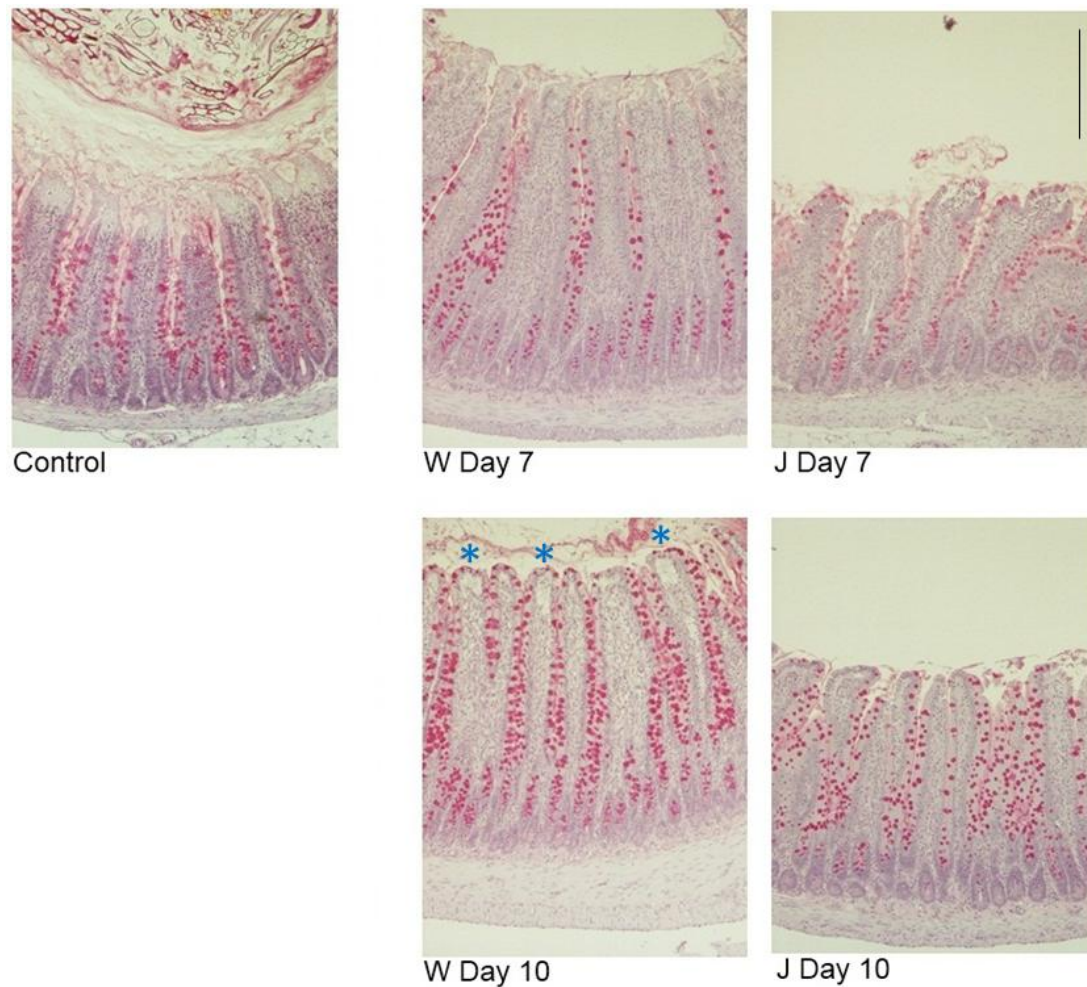
**Figure 3.10. Assessment of parasite fecundity A) by *per capita* egg output *in vivo* and B) *in vitro*.** A: Egg output in faeces (epg) was divided by the number of adults (males + females) residing in the host gut on day 7 post-infection. The error bars show + 1 SEM (where  $n = 5$ , i.e. worm counts recovered from 5 individual animals). A significant difference was observed between the strains, \*  $P < 0.05$ . B: Adult worms (males + females) of the two strains were cultured *in vitro* for 24 hours under standard conditions and egg output measured. Results represent mean egg counts + SEM (where  $n = 5$ ) from 5 separate cultures, each measured in triplicate. A significant difference was observed between the strains, \*\*\*  $P < 0.001$ .

### 3.4. Comparative host response to infection

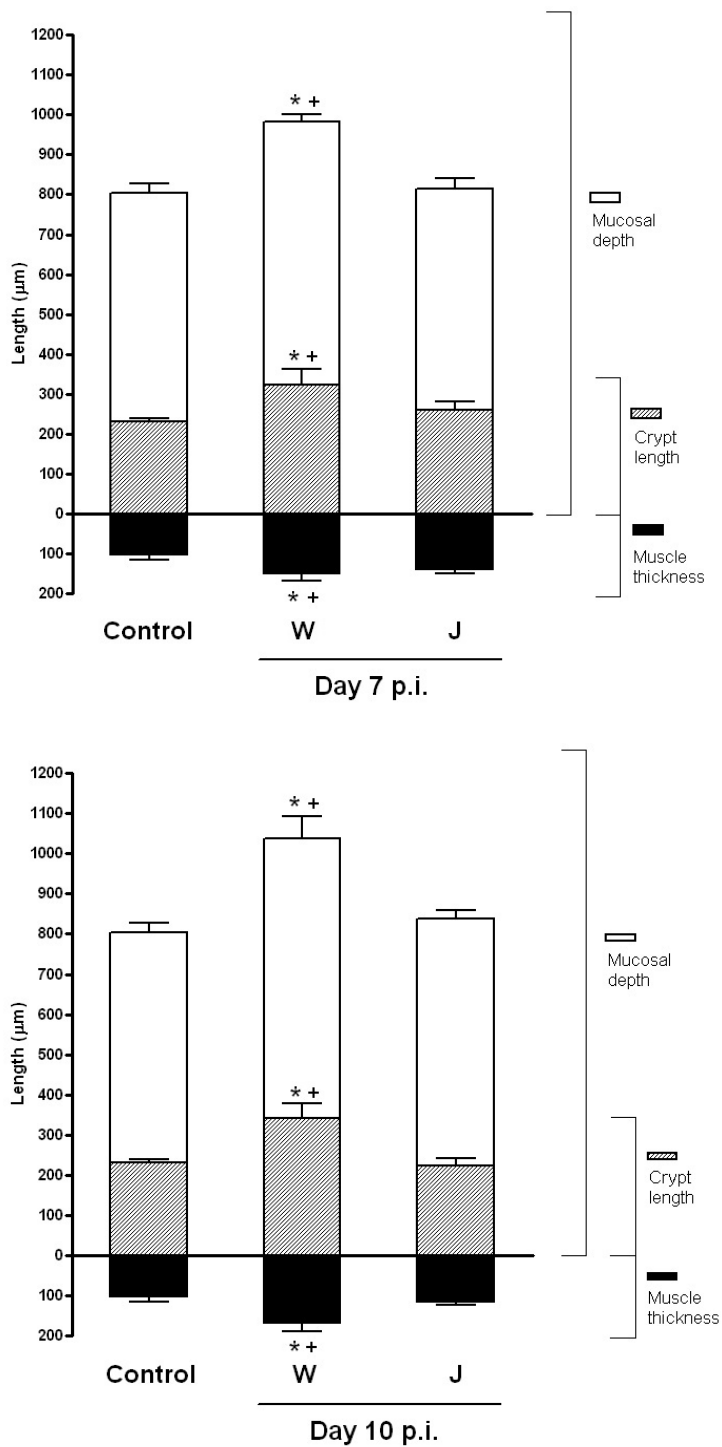
I next sought to investigate whether the differences in infection dynamics could be attributed to a differential host immune response directed against the two strains, using a variety of measures. Morphological changes in the gut epithelium, goblet cell hyperplasia, mastocytosis and lymphocyte activation are all manifestations associated with the host immune response to GI nematodes. These responses were compared between rats infected with the W strain and the J strain of *N. brasiliensis*. In all experiments, rats were infected with 2,000 L3, and uninfected rats matched in sex, age and weight used as controls. Rats were sacrificed on day 7 and day 10 p.i. for assessment of host immune responses.

#### *Morphological changes in jejunum*

To visualise potential changes in gut morphology after infection, cross-sections of rat jejunum were prepared for histology, and representative slides are displayed in Fig. 3.11. Morphological changes in the gut epithelium resulting from an *N. brasiliensis* infection are generally characterised by crypt elongation, increase in muscle thickness, enterocyte detachment and oedema at the villus tips (Perdue, 1989). On observation of the slides, some of these changes were apparent in rats infected with the W strain but not the J strain. On both day 7 and day 10 p.i., crypt length and muscle thickness appeared more prominently increased in rats infected with the W strain than the J strain. For a quantitative assessment, measurements were made of the mucosal depth, crypt length and muscle thickness (Fig. 3.12). Indeed, these measurements showed a small but significant increase in the W strain-infected rats on both day 7 and day 10, in comparison to both the J strain and control. Mucosal depth increased by the same extent as crypt length, and therefore crypt length was increased while villus length remained the same. There was also some evidence of villus tip oedema in rats infected with the W strain on day 10 p.i. (Fig. 3.11, marked by blue asterisks), but this was difficult to assess reliably, as some samples suffered some damage due to flushing of the gut prior to sample fixation. Enterocyte detachment was also difficult to observe for the same reason.



**Figure 3.11. Morphological changes in the jejunum resulting from infection with *N. brasiliensis*.** Jejuna of rats infected with the W strain or the J strain, as well as those of uninfected rats (control), were isolated on day 7 or day 10 post-infection. Slides were prepared and stained with Periodic acid-Schiff (PAS), and photographed at x100 magnification. Sites of heavy villus tip oedema are indicated with blue asterisks (\*). Scale bar (black): 400  $\mu$ m.



**Figure 3.12. Gross alterations in jejunal tissue post-infection with *N. brasiliensis*.** Samples were taken on day 7 or day 10 post-infection from rats infected with the W or the J strain. Each bar represents the mean + 1 SEM (where  $n = 5$ ) of measurements of jejunal tissues harvested from 5 individual rats, in which 20 measurements at regular intervals along the cross-section were taken from each. \*  $P < 0.05$ , relative to uninfected controls. +  $P < 0.05$ , showing a significant difference between the W and J strain.

### ***Goblet cell hyperplasia***

Goblet cell hyperplasia is also a feature of the host response to *N. brasiliensis* infection, and increased mucus secretion contributes to parasite expulsion. The number of goblet cells in latitudinal sections of the jejunum was counted (Fig. 3.13). No significant difference was found in goblet cell numbers on day 7 p.i. with either strain. On day 10 p.i., however, goblet cell numbers in rats infected with the W strain were significantly elevated over uninfected animals, although the difference between the W strain and the J strain did not reach statistical significance.

### ***Mastocytosis***

The systemic release of rat mast cell protease II (RMCP II), a major secreted product of rat mucosal mast cells, was used as an indicator of the extent of mastocytosis. The level of RMCP II in sera of rats infected with both strains was measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 3.14). RMCP II levels were found to be significantly elevated on both day 7 and day 10 p.i. with both strains. A low level of RMCP II was detected in the control (uninfected) group reflecting the basal level of RMCP II secretion. On both day 7 and day 10 p.i., there was no significant difference in RMCP II levels between rats infected with the W and J strain. Therefore, infection with both strains resulted in increased mastocytosis, but this did not differ between the strains.

### ***Cellular responses in the mesenteric lymph nodes (MLN)***

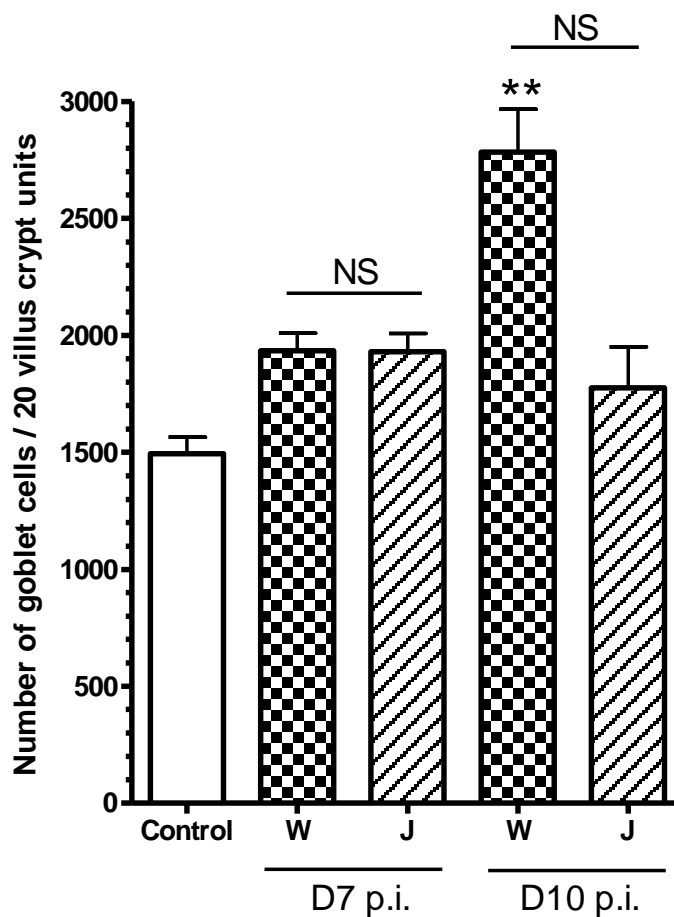
Cells from the draining mesenteric lymph nodes (MLN) were isolated to assess the degree and type of immune response primed by infection. On day 7 p.i., cell numbers were significantly increased in all infected rats, and remained high at day 10 with no significant changes (Fig. 3.15). The cell numbers between the W and J strain were not significantly different on either day, suggesting that both strains induced a similar level of cellular infiltration. MLN cells were then assessed for antigen-specific lymphocyte activation by <sup>3</sup>H-thymidine incorporation (Fig. 3.16). *N. brasiliensis* adult



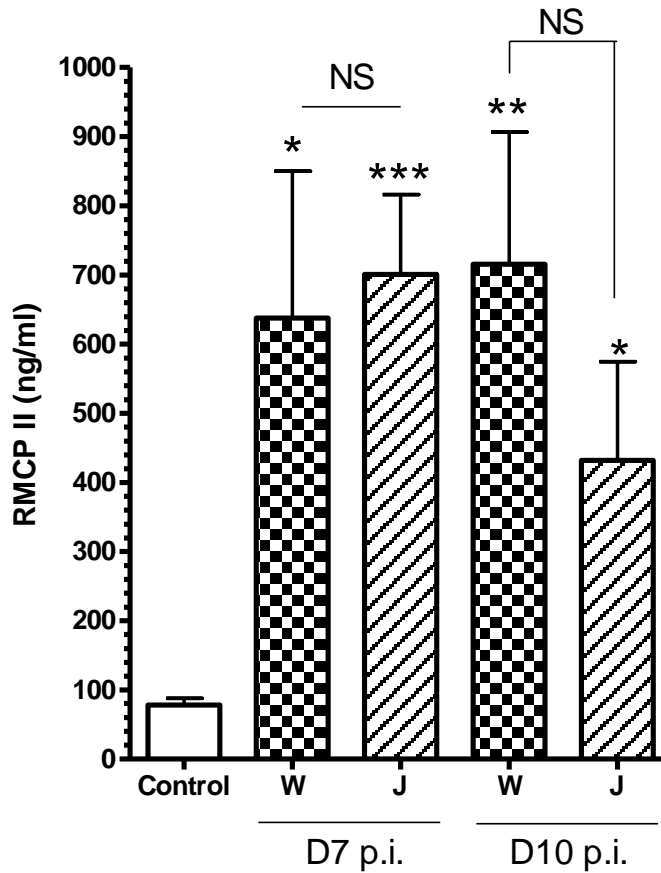
extracts did not stimulate lymphocyte proliferation to a significant level in MLNs harvested on day 7 p.i. for either strain, but showed high levels of activation by day 10 p.i. for both strains. There were no significant differences between infection with either strain on either day, indicative of a similar degree of lymphocyte activation.

Supernatants from the cell cultures were collected to analyse cytokine responses (Fig. 3.17). MLN cells primed by infection with both strains released high levels of IL-10 and IL-13, and moderate levels of IL-4 and IL-5 on stimulation with parasite extracts. These increases could be detected from day 7 p.i., and levels of IL-4, IL-5 and IL-10 were additionally increased by day 10. No IL-12 release was detected, and IFN- $\gamma$  levels were not significantly different from controls on day 7 or 10. These results are consistent with a Th2-biased cytokine profile. No significant differences were found in the cytokine profiles elicited by infection with either strain.

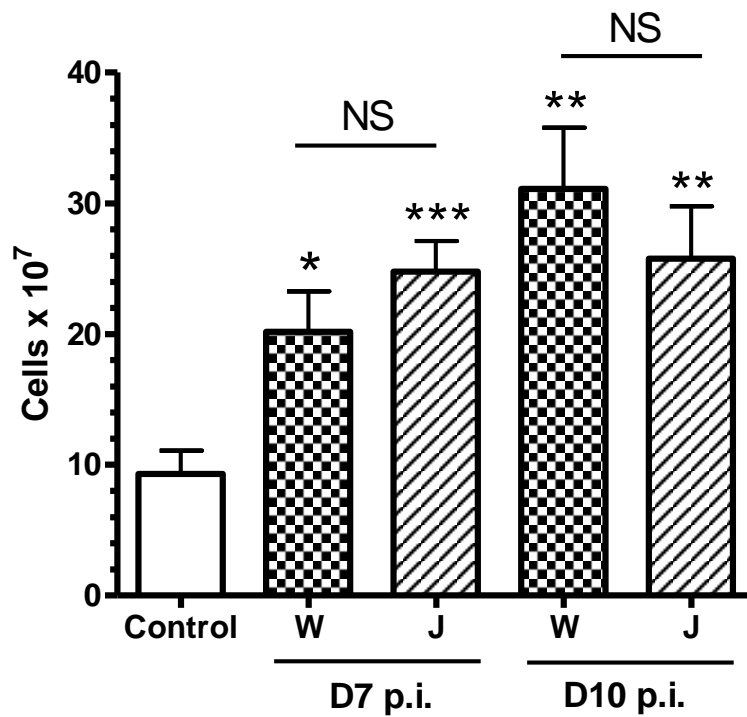
In summary, although infection with the W strain appeared to have elicited slightly greater gross alterations in jejuna tissues than the J strain, this was not supported by the levels of goblet cell hyperplasia, mastocytosis, cellular response and cytokine release by MLN cells, which were stimulated to similar levels by infection with the two strains. It was thus determined that the degree and type of immune response to the two strains were largely similar.



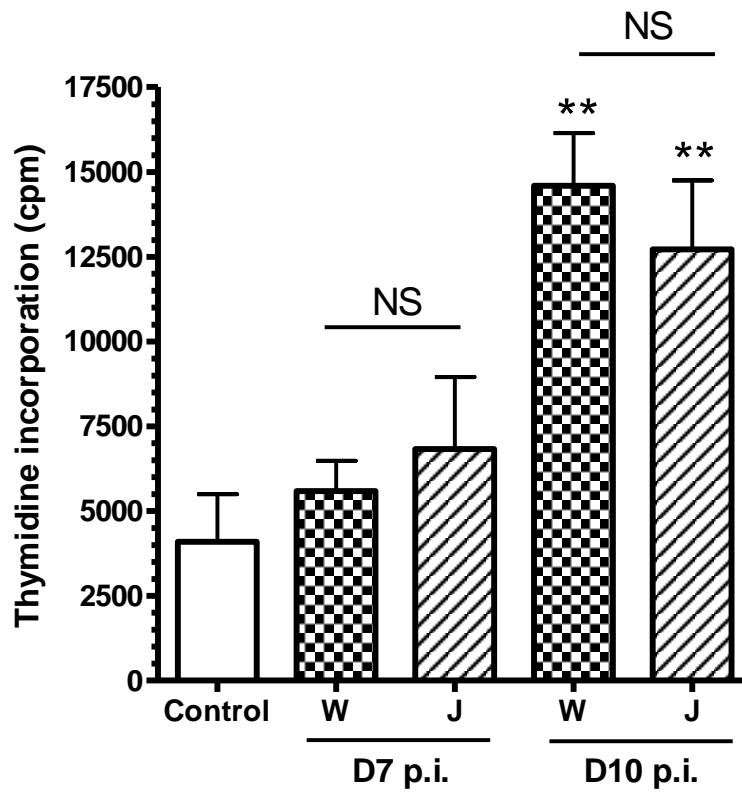
**Figure 3.13. Quantification of goblet cells in the jejunum of infected rats.** Jejunum samples were taken from day 7 or 10 post-infection with the W or J strain of *N. brasiliensis*. Each bar represents the mean + 1 SEM of goblet cells in 20 villus crypt units in jejunum sections (where  $n = 5$  rats). \*\*  $P < 0.01$ , relative to uninfected controls. NS denotes no significant difference between the strains.



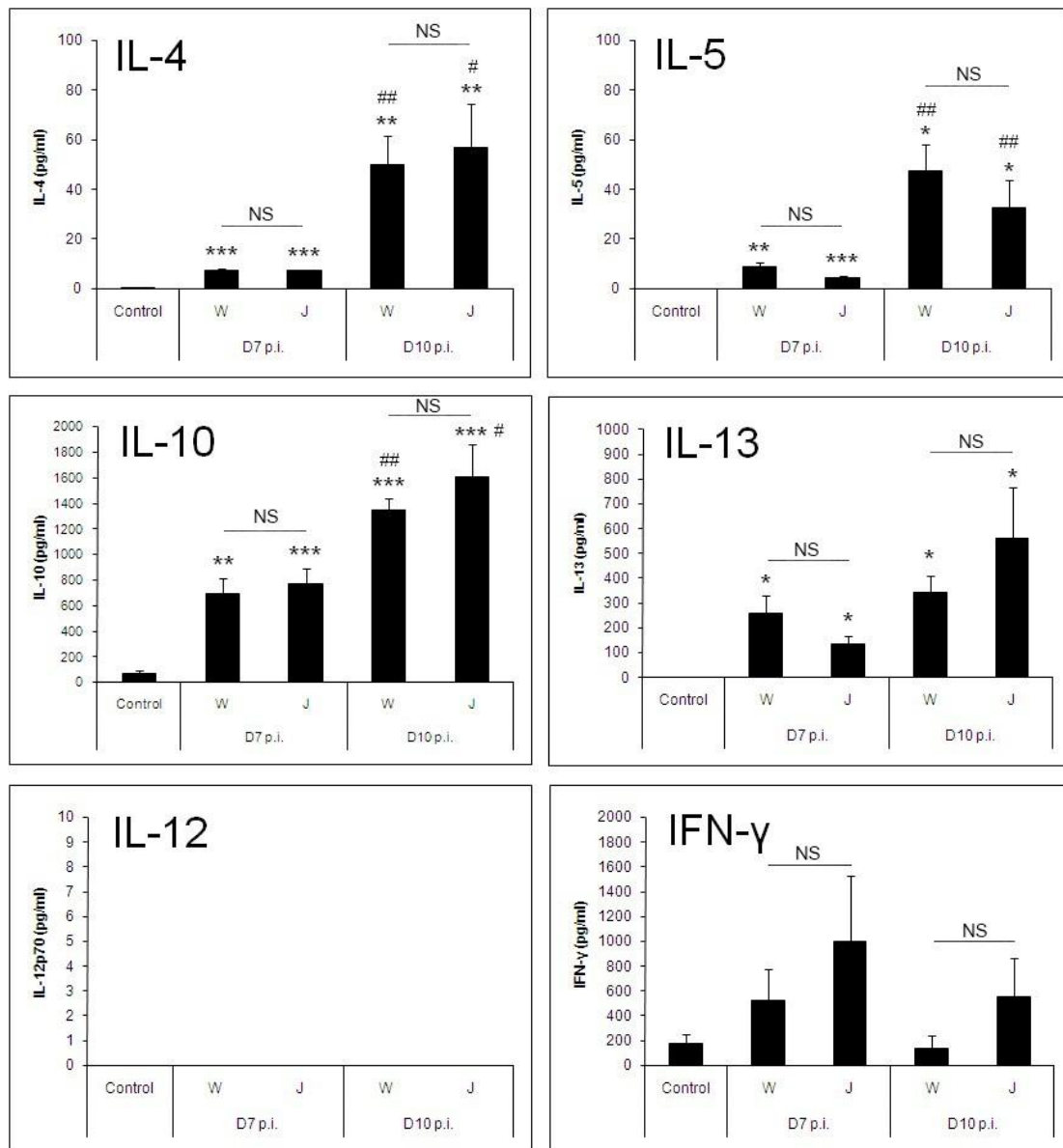
**Figure 3.14. *N. brasiliensis* strains induce comparable levels of mastocytosis.** Serum samples were taken from day 7 or 10 post-infection with the W or J strain for detection of rat mast cell protease II (RMCP II). Each bar represents the mean RMCP II level detected in sera of 5 rats assayed in triplicate, + 1 SEM (where  $n = 5$ ). \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ , relative to uninfected controls. NS represents no significant difference between the strains.



**Figure 3.15. Cellular infiltration into mesenteric lymph nodes (MLN) after infection with *N. brasiliensis*.** Cells were isolated and quantified from the MLNs of rats infected with either strain on day 7 or 10 post-infection, and compared to those from uninfected controls. Figures represent the mean number of cells + 1 SEM (where  $n = 5$ ) from 5 rats. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ , relative to control. NS denotes no significant difference between the strains.



**Figure 3.16. Antigen-specific proliferation of MLN cells.** Cells were isolated from the MLNs of rats infected with the W or J strain, on day 7 or 10 post-infection, and from uninfected rats as controls. Results are expressed as the mean counts per minute (cpm) + 1 SEM (where  $n = 5$ ) of values from cells harvested from 5 rats, each assayed in triplicate. \*\*  $P < 0.01$ , relative to control. NS represents no significant difference between the strains.



**Figure 3.17. Antigen-specific cytokine responses.** Cells from the MLNs were harvested from rats infected with the W or J strain of *N. brasiliensis* on day 7 or 10 post-infection. Bars show means + 1 SEM (where  $n = 5$ ) of cytokines secreted by cells from 5 rats, each assayed in triplicate. Asterisks (\*) show a significant difference relative to the control, and hash (#) signs indicate a significant elevation of cytokine secretion on day 10 relative to day 7 post-infection of the given strain. Three symbols:  $P < 0.001$ , two symbols:  $P < 0.01$ , one symbol:  $P < 0.05$ . NS represents no significant difference between the strains.

### 3.5. Comparison of secreted proteins

Since the degree and type of immune response generated by infection with the two strains was largely similar, it was possible that differences in persistence and fecundity were related to intrinsic properties of the parasite, perhaps more importantly at the adult stage. Secreted proteins constitute a major form of communication from the parasite to the host, and have the potential to influence the infection in a myriad of ways such as invasion, evasion and modulation of host responses etc. A comparative analysis of parasite secreted proteins was therefore performed.

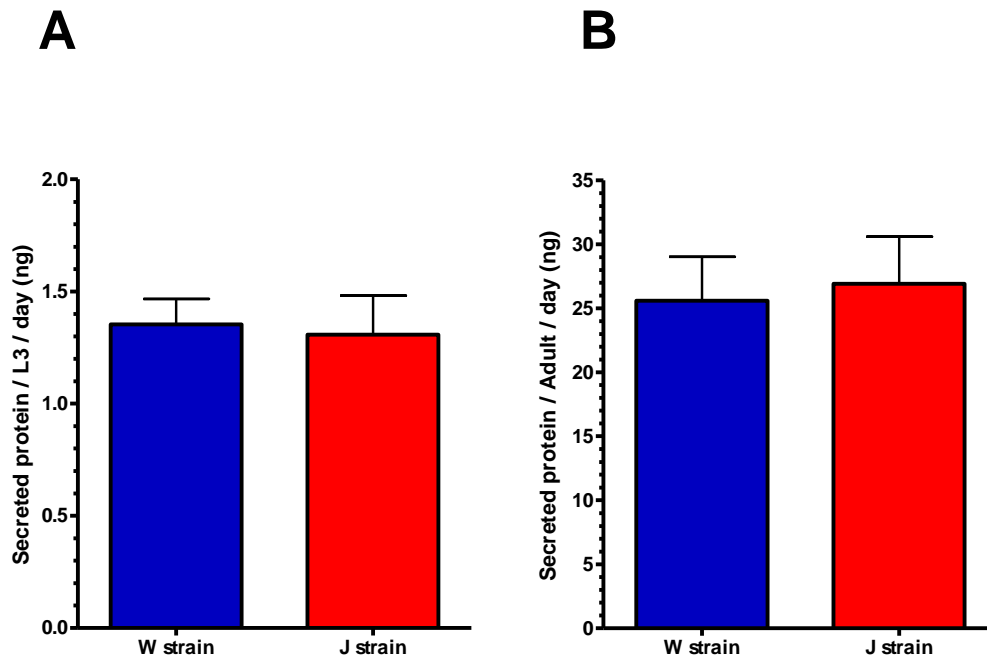
The amount of protein secreted by activated L3 (L3A, activated by culture at 37°C) and adults of the W and J strain of *N. brasiliensis* were compared (Fig. 3.18), and it was found that the two strains secrete similar amounts of proteins. The amount of proteins secreted per day of culture was approximately 1.3 ng per L3A, and 26 ng per adult worm. The majority of L3A secreted proteins lie between a molecular weight of 15-37 kDa, with some subtle differences between the strains evident by one-dimensional (1-D) gradient SDS-PAGE (Fig. 3.19A). The 1-D resolution of L3A secreted proteins between the 15-20 kDa region generally appears as a smear rather than distinctive bands, the effect of which was seen in all batches of culture tested. This suggests that there may be a large variety of proteins secreted in small quantities within this molecular weight range, which was confirmed by two-dimensional gel electrophoresis (2-DE) (Fig. 3.20), on which large numbers of small protein spots can be resolved around this region. The composition of L3A secreted proteins of the two strains was found to be largely similar. By Coomassie staining, approximately 80-100 individual protein spots could be visualised, all of which are below 37 kDa. The precise number would need to be confirmed by gel imaging software, which may also be able to pick up subtle differences in the secreted protein profiles of the two strains.

In contrast, the composition of adult secreted proteins show a broader range in molecular weight, as shown by 1-D SDS-PAGE (Fig. 3.19B). Distinctive bands can be seen, suggesting that particular proteins are secreted in large amounts. Resolution by 2-DE showed that there are indeed many prominent protein spots (Fig. 3.21). Several proteins were resolved at the same molecular weight but with different isoelectric points (*pI*), perhaps suggesting differences in glycosylation or other kinds

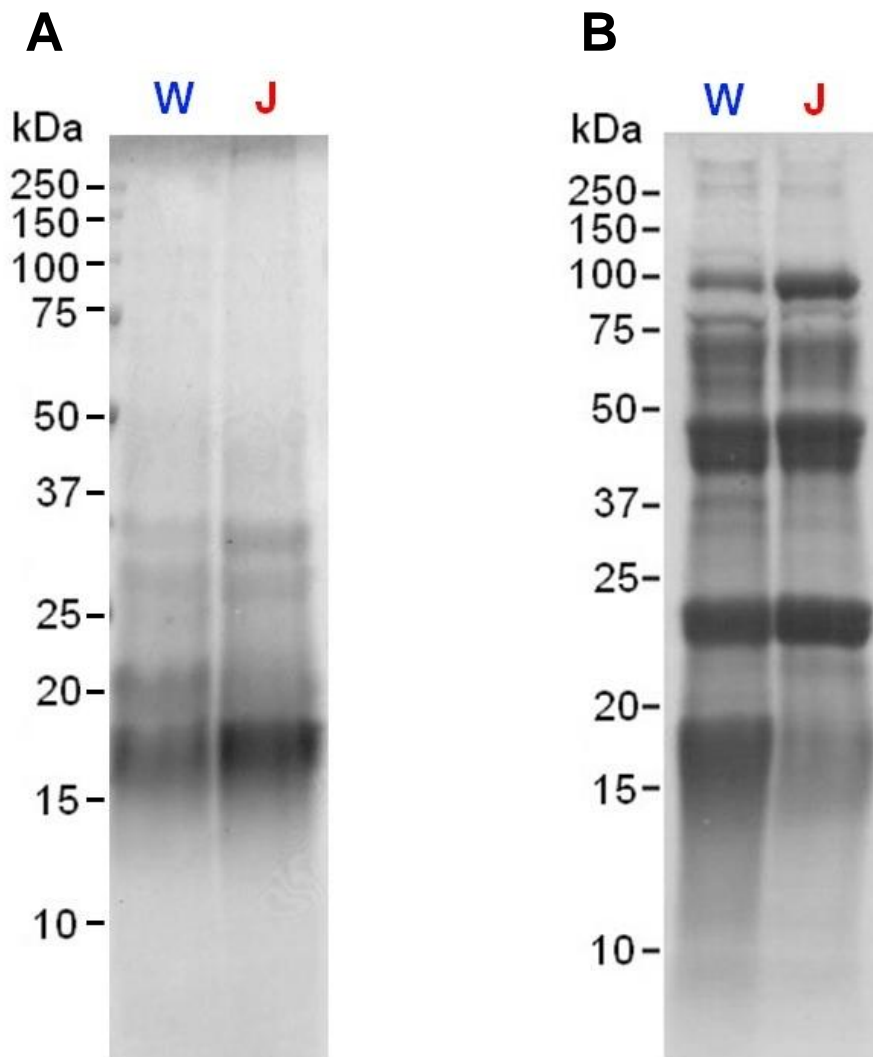
of post-translation modification of the same protein. Approximately 140-160 protein spots can be visualised by eye, which suggests that secreted proteins of adults are more complex than those of activated L3. The separation profile of adult secreted proteins was again largely similar between the two strains, but some differences could be seen. Elucidation of the identity of these proteins may perhaps be helpful in explaining the differences in infection dynamics of the two strains.

The 2-DE separation profiles shown in Fig. 3.19 and Fig. 3.20 represent the majority of proteins secreted by L3A and adults, as these were confirmed by gel separation of radiolabelled proteins and visualisation by autoradiography. However, it is possible that some proteins are secreted in such small amounts that could not be visualised by either method. Although silver staining is less sensitive than radioactive detection, it is more sensitive than Coomassie staining, and would be useful for proteomic work in future studies.

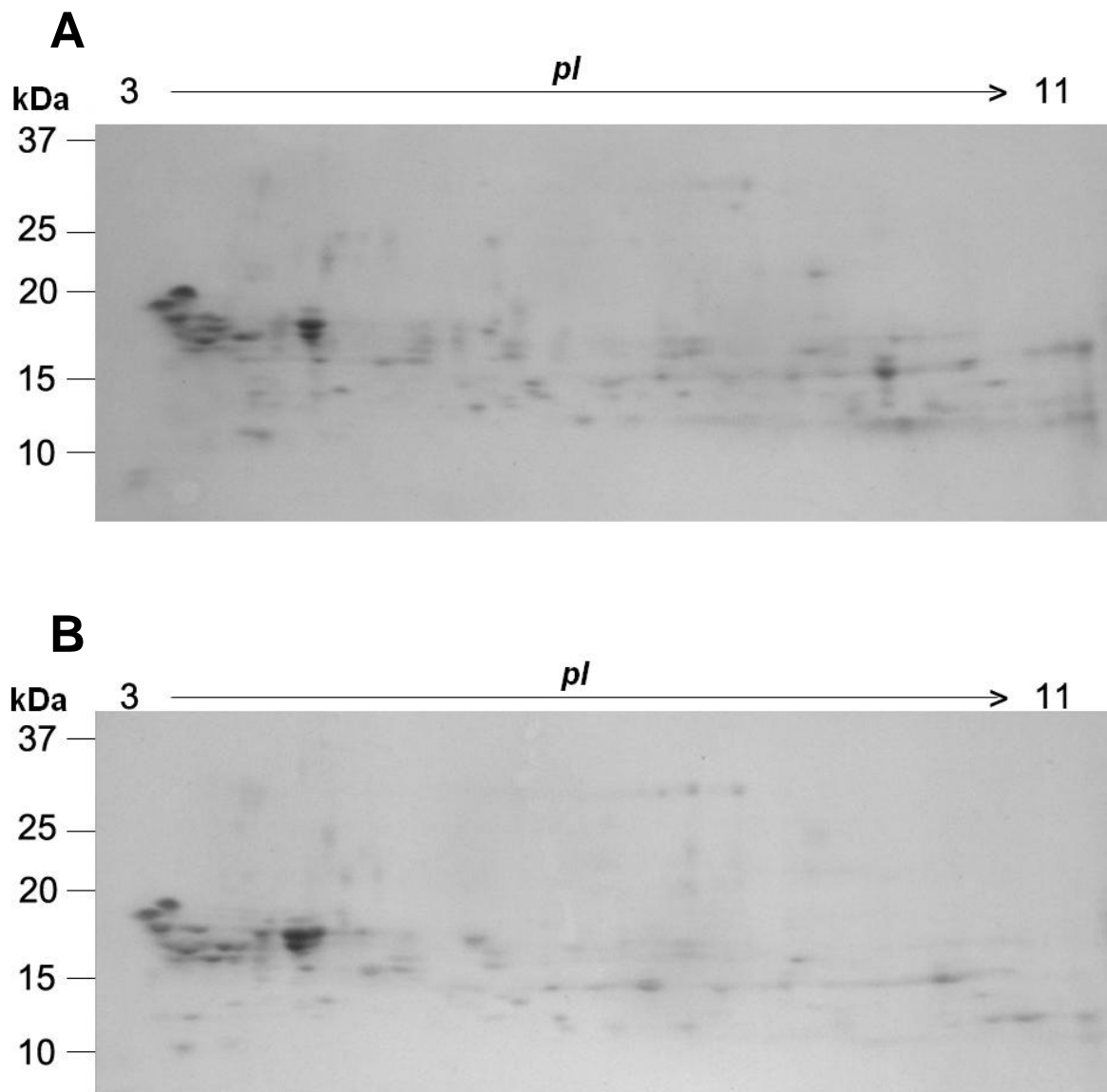




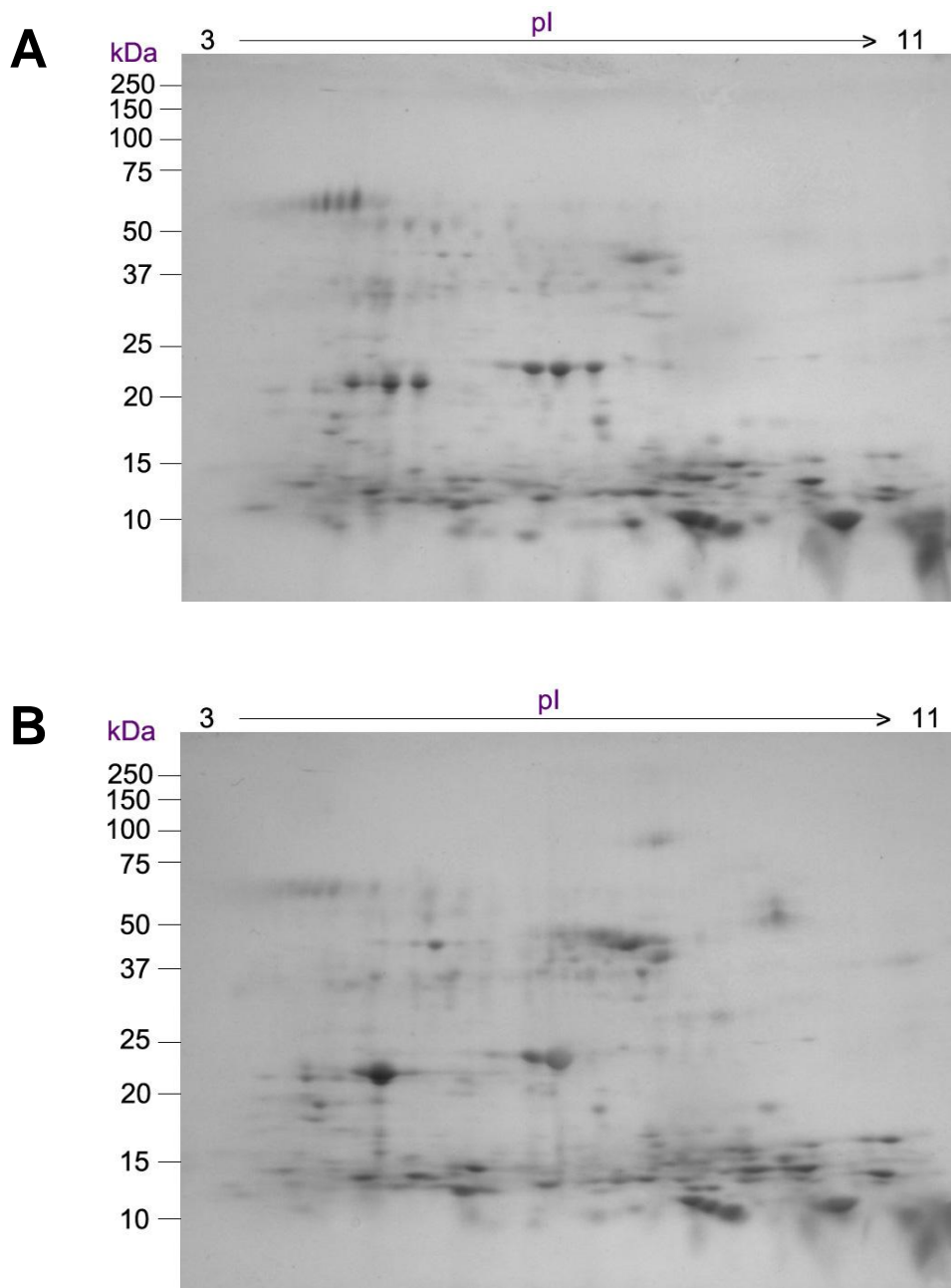
**Figure 3.18. The two strains of *N. brasiliensis* secrete similar amounts of proteins.** Parasites were cultured *in vitro* under standard conditions, and secreted proteins collected from the medium and quantified for L3A (A) and adults (B). Results are expressed as the mean amount of protein secreted per parasite per day + 1 SEM (where  $n = 5$ ), from 5 individual cultures assayed in triplicate.



**Figure 3.19. The secretion profiles of *N. brasiliensis* strains resolved by 1D gradient SDS-PAGE.** Proteins secreted by A) L3A and B) adults were collected after 3 days of culture and concentrated. Approximately 60  $\mu\text{g}$  of proteins were resolved by 10-20% gradient SDS-PAGE and stained with Coomassie blue.



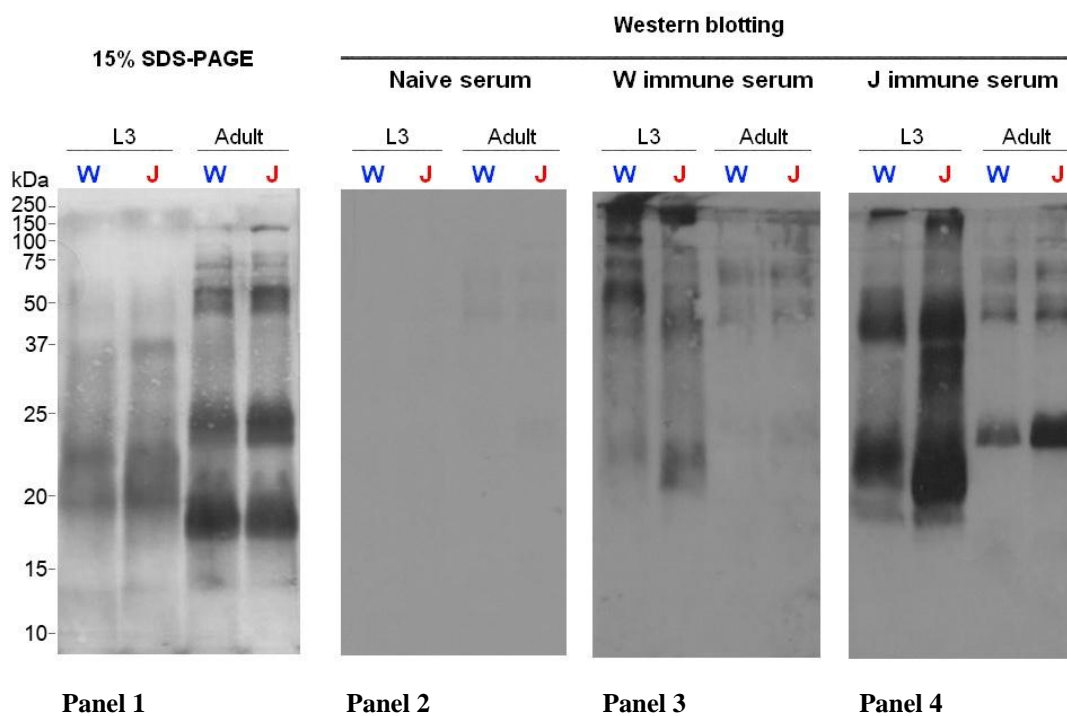
**Figure 3.20. Proteins secreted by activated L3A of each strain are largely similar.** Proteins were separated by 2DE; horizontally by isoelectric focusing then vertically on a 10-20% polyacrylamide gradient gel, and stained with Coomassie blue. Each gel was loaded with 170  $\mu$ g of secreted protein. Panel A: W strain; Panel B: J strain.



**Figure 3.21. Proteins secreted by adult worms show subtle differences.** Proteins were separated by 2DE; horizontally by isoelectric focusing, then vertically on a 10-20% polyacrylamide gradient gel, and stained with Coomassie blue. Each gel was loaded with 170  $\mu\text{g}$  of secreted protein. Panel A: W strain; Panel B: J strain.

### ***Immunogenicity***

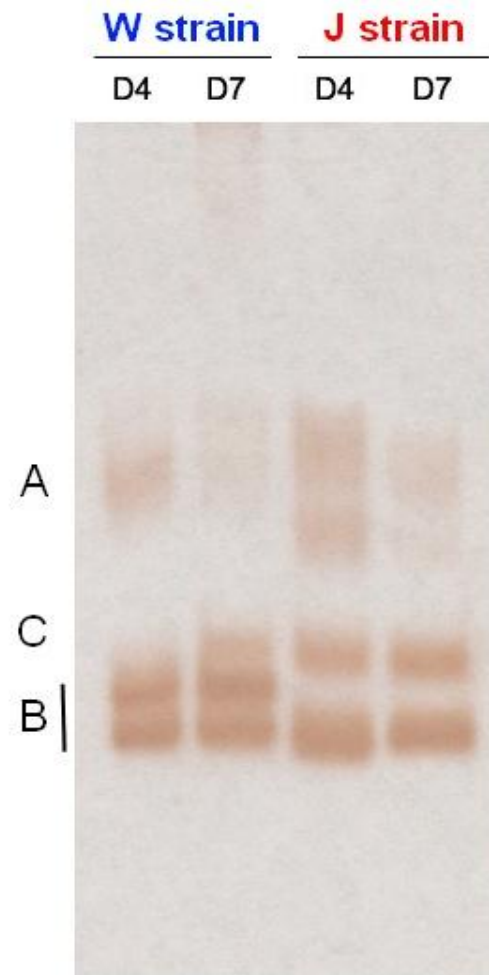
Differential recognition of proteins secreted by the two strains of *N. brasiliensis* was analysed by Western blotting using immune sera collected from rats infected twice with either the W or J strain as a probe (Fig. 3.22). Immune serum to the J strain was able to bind to proteins secreted by the W strain, and vice versa, indicating cross-recognition. Strain-specific reaction appeared to be stronger, that is, the J strain immune serum shows stronger reactivity with J strain-secreted proteins than with W strain secreted proteins (Panel 4, comparison between W and J lanes). Overall, the J strain immune serum reacted more strongly with every protein sample than did the W strain immune serum (comparison between panels 3 and 4), perhaps reflecting a stronger antigenic stimulus resulting from more persistent infection. Proteins secreted by L3A reacted more strongly with immune sera than that of adults, probably as a result of the reinfection process, during which the parasites are eliminated before they reach the adult stage, so that only L3-specific factors were re-exposed to the immune system, stimulating the memory response and expansion of L3-specific antibodies.



**Figure 3.22. Reactivity of proteins secreted by the two strains with immune sera.** Proteins secreted by L3A and adults of the two strains were resolved by 15% SDS-PAGE and reacted with immune sera collected from rats infected twice with either the W or J strain. Panel 1 shows the protein separation profile of 15  $\mu$ g L3A or adult secreted proteins of the two strains, visualised by DB71 staining. Duplicate gels were blotted onto nitrocellulose membranes and reacted with W or J strain immune sera (panels 3 and 4 respectively), or with naive rat serum as a negative control (panel 2). Sera (pooled from 5 individual rats for each condition) were used as a primary antibody at a dilution of 1:500, followed by anti-rat IgG-HRP secondary antibody at 1:60,000.

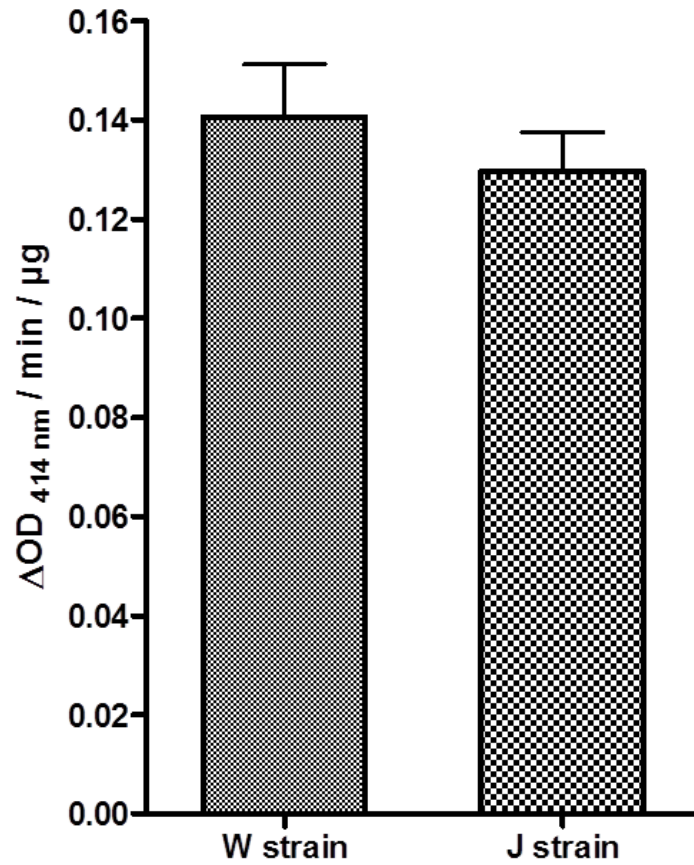
### ***Secretion of acetylcholinesterase (AChE)***

Acetylcholinesterase (AChE) is one of the major known secretory enzymes of *N. brasiliensis*. It is thought to be an important molecule in modulation of immunity and parasite persistence (Selkirk *et al.*, 2005), so secretion of AChE by adult worms was analysed. It is known that young adults of *N. brasiliensis* secrete three isoforms of AChE (isoform A, B and C), and they switch to secreting only isoform B and C as they mature (Hussein *et al.*, 2002). Isoform B is visualised as two distinctive bands, and previous studies have shown that this is due to proteolytic cleavage of AChEs (Selkirk, personal communication). It has been proposed that isoform switching of AChE during different life cycle stages is a strategy to evade action of antibodies directed against the earlier forms of the enzyme (Lee, 1996), although this seems unlikely given their sequence similarity. By analysing the secreted proteins of adults isolated on day 4 p.i. (young adults) and day 7 p.i. (mature adults), these isoforms were clearly demonstrated in the W strain of *N. brasiliensis*. However, some differences in electrophoretic mobility were observed for AChEs from the J strain compared to the W strain (Fig. 3.23). The downregulation of isoform A from day 4 to day 7 p.i. was still apparent in the J strain, but the distinction of isoforms B and C was unclear. The overall amount of AChE activity in the secreted proteins of the two strains was however similar, suggesting that they are equally competent in metabolism of acetylcholine (Fig. 3.24).



**Figure 3.23. Comparison of acetylcholinesterase secretion by adult worms.** Secreted proteins from day 4 p.i. (young adults) or day 7 p.i. (mature adults) of both strains were resolved by 8% native polyacrylamide gel electrophoresis, and visualised using a direct-colouring thiocholine method for cholinesterases (Karnovsky and Roots, 1964). The isoforms A, C and B are annotated on the left. The bands within the isoform B region represent proteolytically modified forms of the same protein.





**Figure 3.24. Levels of acetylcholinesterase activity in adult secreted proteins.** Adult secreted proteins collected from the two strains were added at 0.5  $\mu\text{g}$  to each reaction, and incubated for 10 minutes. Results are expressed as the mean change in  $OD_{414 \text{ nm}}$  per minute per  $\mu\text{g}$  of *N. brasiliensis* adult secreted protein resulting from 5 secreted protein preparations assayed in triplicate, with error bars representing + 1 SEM ( $n = 5$ ).

### *Secretion of nucleotide metabolising enzymes*

The higher persistence of the J strain may be related to a superior capacity to resist host immune expulsion mechanisms. A possible way in which this may be achieved is through the expression of anti-inflammatory molecules such as the nucleotide metabolising enzymes (NMEs). Enzymatic assays for phosphate hydrolysis were therefore carried out to investigate whether *N. brasiliensis* secretes any enzymes with an ability to metabolise nucleotides, and if they do, whether their activity differs between the strains.

Whole secreted protein samples collected from adult *N. brasiliensis* were tested for hydrolysis of ATP, ADP, AMP and UDP, assaying for liberation of inorganic phosphate. Secreted proteins from both strains of *N. brasiliensis* hydrolysed ATP, ADP and UDP with very high specific activities, but not AMP (Table 3.1). This provides novel data that *N. brasiliensis* is capable of metabolising extracellular nucleotides. Enzymatic activity on the substrate (Na)ATP was higher than that for (Mg)ATP, suggesting that sodium is a more effective cofactor than magnesium in assisting the hydrolytic activities of the enzyme(s) involved. However, enzymatic activity in proteins secreted by the W strain was significantly higher than that of the J strain for all substrates (except (Mg)ATP), which suggests that the greater persistence of the latter cannot be attributed to a higher level of nucleotide-metabolising activities.

The nucleotide-metabolising enzymes of *N. brasiliensis* appear to exhibit classic apyrase activities similar to those from arthropods, which readily hydrolyse ATP and ADP but not AMP. This differs from *T. spiralis*, the secreted proteins of which show no reactivity with ATP, but hydrolyse ADP, UDP and AMP via a single enzyme, 5'-nucleotidase (enzymatic scheme shown in Fig. 3.25; Gounaris *et al.*, 2004). Secreted proteins from both *N. brasiliensis* strains hydrolysed ADP at a higher rate than ATP. If both activities were catalysed by a single apyrase, the ADP released upon ATP hydrolysis should be further broken down into AMP, resulting in a higher amount of phosphate release. There are two plausible explanations for the discrepancies in specific activities for the two substrates: 1) a single apyrase secreted by *N. brasiliensis* is much more efficient in hydrolysing ADP as a substrate than ATP, creating a rate-

limiting step which resulted in a slower overall reaction; or 2) the two reactions are catalysed by separate enzymes.

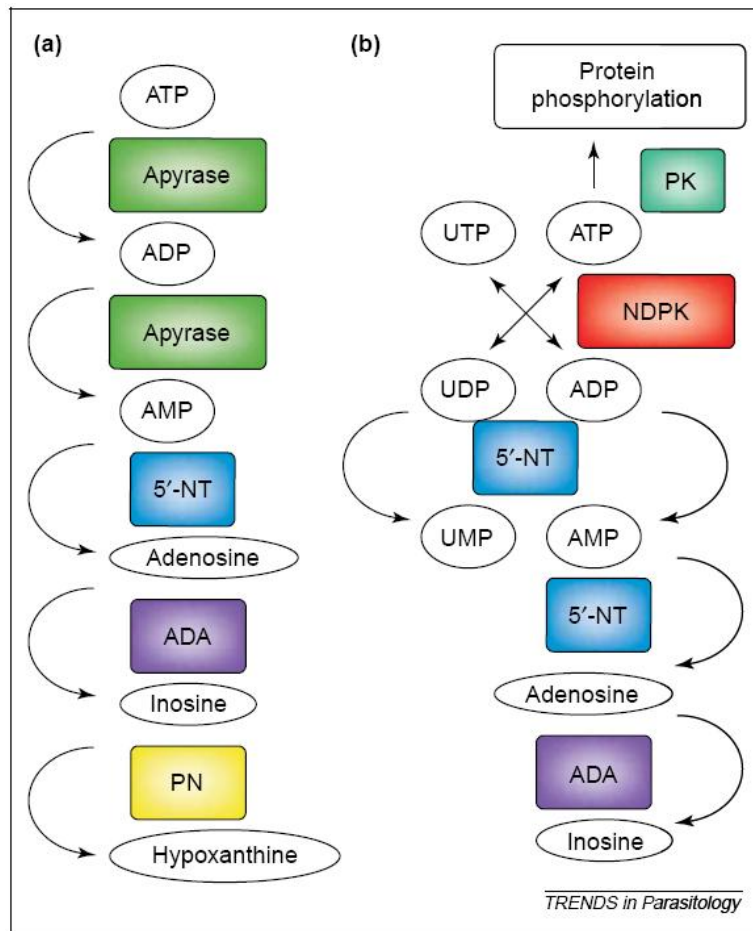
Since the substrate specificity of the NMEs of *N. brasiliensis* differs from that of *T. spiralis*, there is a further consideration that the action of these enzymes may not be exclusive to nucleotide hydrolysis. To test the substrate specificity of the reaction, sodium pyrophosphate and glucose-6-phosphate (G6P) were used instead of the nucleotides. It was found that sodium pyrophosphate could indeed be hydrolysed by *N. brasiliensis* adult secreted proteins, but no such activity was found against G6P (Table 3.2). This may suggest that enzyme(s) present in *N. brasiliensis* secreted products can cleave the bond between two linked phosphates, but not a phosphate residue linked to another (non-phosphate) moiety, such as AMP and G6P. Furthermore, they also appear to be more adept at cleaving phosphate from diphosphates (e.g. ADP) than triphosphates (e.g. ATP).

The activities observed could debatably have been catalysed by a non-specific phosphatase. Therefore, in the next sets of experiments, phosphatase inhibitors were used to test if they blocked the reactions (Table 3.3). Levamisole did not affect the ability of secreted proteins in hydrolysing ATP, ADP or sodium pyrophosphate, suggesting that alkaline phosphatases were not responsible for the reactions. In contrast, sodium fluoride (NaF) was found to completely inhibit liberation of phosphate from all three compounds. Although NaF has been shown to inhibit the activities of both 5'-NT and apyrase, it is also an inhibitor to acid phosphatases, which have an acidic pH optimum. Assays were therefore performed at pH values ranging from 6.5 to 9.5. Hydrolysis of ATP and ADP occurred with a pH optimum of 8.5-9.5 (Fig. 3.26), suggesting that the reactions were not effected by an acid phosphatase.

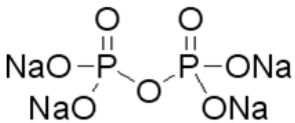
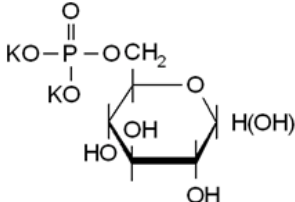
In summary, these experiments show that secreted proteins from *N. brasiliensis* adult worms contain enzymes capable of hydrolysing extracellular nucleotides with high efficiency via a pathway which appears to differ from *T. spiralis*, although it is difficult to come to firm conclusions when dealing with a preparation (total secreted proteins) which may contain multiple enzymatic activities. Nevertheless, these results do not explain the higher persistence of the J strain of *N. brasiliensis*.

<u>Activity</u>	<u>Substrate</u>	<u>Specific Activity</u> (nmol/min/mg secreted protein)	
		<u>W strain</u>	<u>J strain</u>
ATPase	(Mg)ATP	4984 ± 194	3326 ± 312
	(Na)ATP	8668 ± 408	4709 ± 389 *
ADPase	ADP	11435 ± 518	6864 ± 663 *
UDPase	UDP	13041 ± 306	5685 ± 238 *
AMPase	5'-AMP	not detected	not detected

**Table 3.1. Enzymatic activities in *N. brasiliensis* adult secreted proteins involved in the metabolism of nucleotides.** Three individual protein samples from each strain were assayed in triplicate, and the results are expressed as mean ± 1 SEM. All substrates were used at 2.5 mM. *N. brasiliensis* whole secreted proteins were used at a final concentration of 3.75 ng/μl (0.15 μg was added in a 40 μl reaction) for the substrates ATP, ADP and UDP. AMPase activities were tested using up to 5 μg of *N. brasiliensis* whole secreted proteins at various pH values. Values which differ significantly between the strains are indicated \*  $P < 0.05$ .



**Figure 3.25. Schemes for nucleotide metabolising enzymatic cascades in (a) haematophagous arthropods; and (b) *T. spiralis*.** Abbreviations: 5'-NT: 5'-nucleotidase; ADA: adenosine deaminase; NDPK: nucleoside diphosphate kinase; PK: exo- and ecto-protein kinases, PN: purine nucleotidase. Figure is from Gounaris and Selkirk (2005).

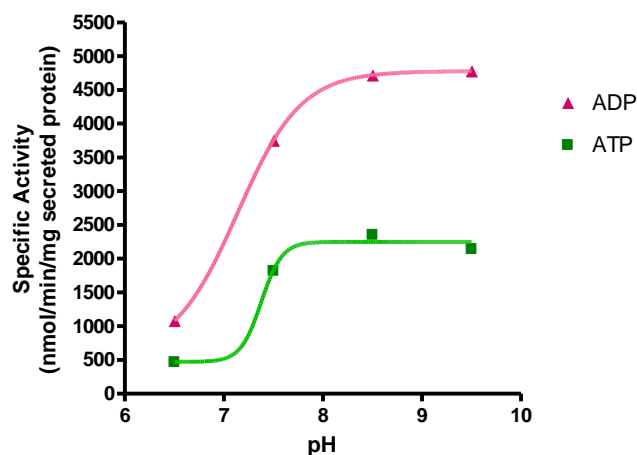
Sodium Pyrophosphate	Glucose-6-phosphate
	

<u>Substrate</u>	<u>Specific Activity</u> (nmol/min/mg secreted protein)	
	<u>W strain</u>	<u>J strain</u>
Sodium pyrophosphate	633 ± 27	532 ± 12
Glucose-6-phosphate	Not detected	Not detected

**Table 3.2. Liberation of phosphate from other substrates by *N. brasiliensis* secreted proteins.** Both sodium pyrophosphate and glucose-6-phosphate were used at a final concentration of 5 mM. *N. brasiliensis* whole secreted proteins were used in the reaction at a final concentration of 25 ng/μl (1 μg was used in a 40 μl reaction). Results are expressed as means ± 1 SEM. The chemical structure of the substrates are shown at the top.

<u>Inhibitor</u>	<u>Inhibition of phosphate release from substrate</u>		
	<u>ATP</u>	<u>ADP</u>	<u>Sodium Pyrophosphate</u>
<b>Levamisole</b>	No effect	No effect	No effect
<b>NaF</b>	Complete inhibition	Complete inhibition	Complete inhibition
<b>NaF + Levamisole</b>	Complete inhibition	Complete inhibition	Complete inhibition

**Table 3.3. Effect of phosphatase inhibitors on phosphate-hydrolysing activities of *N. brasiliensis* secreted proteins.** The inhibitors, levamisole and sodium fluoride (NaF), were included at a final concentration of 5 mM. ATP and ADP substrates were used at 2.5 mM, and sodium pyrophosphate was used at 5 mM final concentration. *N. brasiliensis* whole secreted proteins were used in the reaction at a final concentration of 3.75 ng/ $\mu$ l (0.15  $\mu$ g was added to a 40  $\mu$ l reaction) for the substrates ATP and ADP, and at a final concentration of 25 ng/ $\mu$ l (1  $\mu$ g was added to a 40  $\mu$ l reaction) for sodium pyrophosphate.



**Figure 3.26. Phosphatase activities in *N. brasiliensis* secreted protein showed an alkaline pH optimum.** ATP and ADP substrates were used at a final concentration of 2.5 mM. *N. brasiliensis* (W strain) whole secreted proteins were used in the reaction at a final concentration of 3.75 ng/ $\mu$ l (0.15  $\mu$ g was added to a 40  $\mu$ l reaction). Assays were performed at pH values ranging from 6.5 to 9.5, with bis-tris propane substituting hepes as the reaction buffer for its wider buffering range.

### 3.6. Infection dynamics of the Japanese strain after multiple laboratory passages

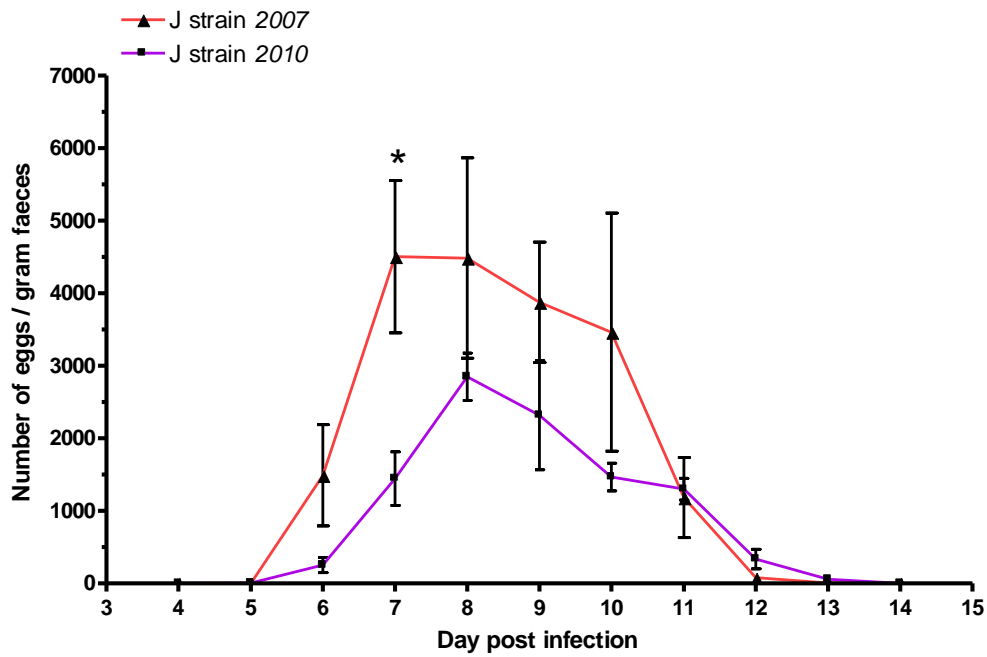
The comparison of infection dynamics between the W and J strain in section 3.3 aimed to study changes resulting from multiple passage in a laboratory setting over a long period of time. A flaw in this comparison is that the two strains originated from different geographical locations, and might have intrinsically different properties. By the year 2010, I performed serial passage of the J strain through 30-40 generations in SD rats for maintenance of the life cycle. This presented an opportunity to carry out an analysis of the effect of laboratory passage on the same strain.

The overall dynamics of establishment and expulsion of adult worms was not changed after three years of laboratory passage of the J strain (Fig. 3.27), but egg output was significantly diminished (Fig. 3.28). The total egg output over a primary infection was  $19,085x$  (where  $x$  is the amount of faeces produced by a rat per day in grams) in year 2007, and only  $10027x$  in year 2010, which is an overall reduction of 47.5%. The *per capita* fecundity on day 7 p.i. was  $6.47x$  per worm in 2007, and  $2.61x$  in 2010 (Fig. 3.29A). Adults isolated on day 7 p.i. were also isolated and cultured for an assessment of fecundity *in vitro* (Fig. 3.29B), which was found to be significantly decreased in 2010 compared to 2007 by over 28%. Collectively, these data provide strong evidence that multiple laboratory passage results in a loss of fecundity, and ultimately total egg output, of a parasite strain, although no differences in persistence or survival was seen in this particular experiment.

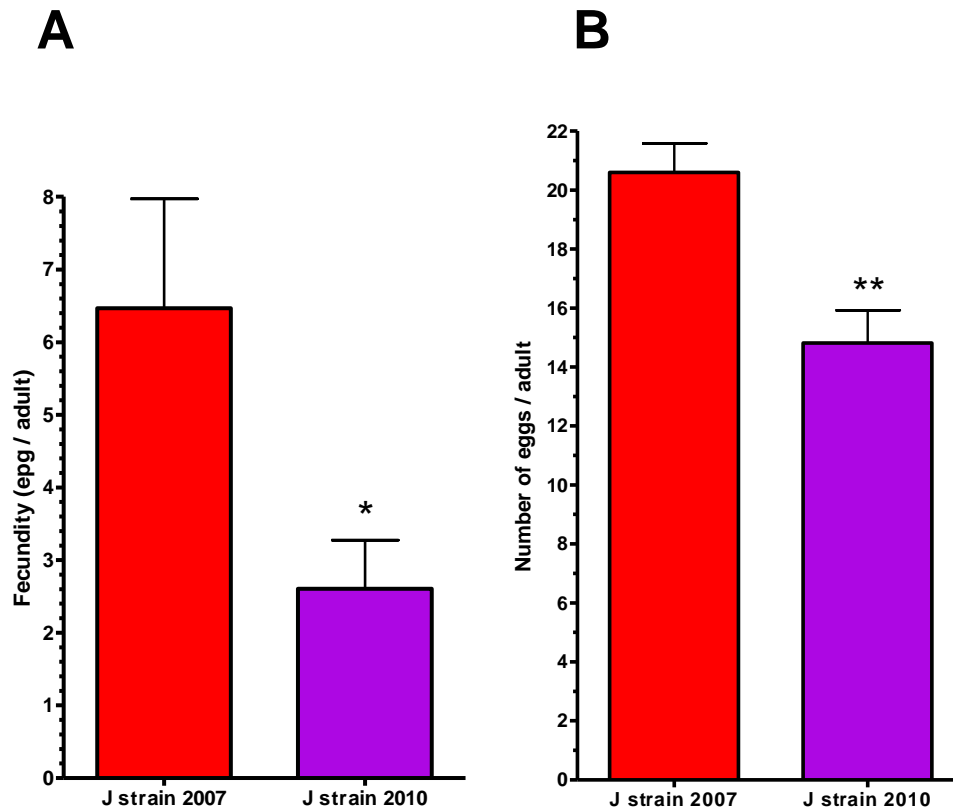




**Figure 3.27. Dynamics of establishment and expulsion of adult worms before and after laboratory passage.** Rats were infected with 2,000 J strain L3 in 2007 (red bars) and 2010 (purple bars), and adults were recovered from the intestines at the indicated days post-infection. Results are presented as the mean number of worms recovered + 1 SEM (where  $n = 5$  rats per group). No significant difference was observed between the groups on any day of sampling.



**Figure 3.28. Faecal egg output before and after laboratory passage.** Points represent the mean number of eggs recovered per gram of faeces from rats infected with 2000 J strain L3 in 2007 or 2010. Counts were performed daily from day 3 to day 14 post-infection. Error bars indicate  $\pm 1$  SEM ( $n = 5$  rats per group). \* $P < 0.05$ .



**Figure 3.29. Assessment of parasite fecundity before and after laboratory passage A) by *per capita* egg output *in vivo* and B) *in vitro*.** A: Egg output in faeces (epg) was divided by the number of adults (males + females) residing in the host gut on day 7 post-infection. A significant difference was observed, \*  $P < 0.05$ . B: Adult worms of the two strains were cultured *in vitro* for 24 hours under standard conditions and egg output measured. Results represent mean egg counts + SEM (where  $n = 5$ ) from 5 separate cultures, each measured in triplicate. A significant difference was found, \*\*  $P < 0.01$ .

### 3.7. Discussion

The objective of this chapter was to examine the effects of laboratory passage on *N. brasiliensis* in terms of infection dynamics, host response to infection, and secreted proteins. The implications of these findings will be discussed in this section.

#### *The use of rDNA regions in distinguishing species*

Comparison of rDNA sequences provided strong evidence to confirm the identity of the J strain as *N. brasiliensis*, as both its ITS-1 and ITS-2 regions were completely identical with the W strain, and different from very closely related nematodes such as *C. minutus*. The use of these sequences to distinguish between sister species is a widely accepted technique in the nematode community (Audebert *et al.*, 2005; Blaxter *et al.*, 1998; Dorris *et al.*, 1999), and in a study of *T. muris*, ITS-2 sequences were also used to confirm the identity of a field isolate, which was 100% identical to laboratory passaged strains (Johnston *et al.*, 2005). ITS-2 is a particularly useful rDNA region in the discrimination of species, as it is highly polymorphic between different species, but usually conserved within the same species (Dorris *et al.*, 1999; Muller *et al.*, 2007). Ultimately, the definition of a species is based on the ability to reproduce intraspecifically (Mayr, 1982). It was found across various eukaryotic systems that when the evolutionary distance of two taxa accumulated to a point at which even one compensatory base change in certain regions of the ITS-2 sequences occurred, populations in these two taxa were incapable of intercrossing. This concept has been extended with a classifier that a taxa difference of at least one base gives a 93.11% reliability to indicate that the organisms belong to different species (Muller *et al.*, 2007). Therefore, the lack of variation of the ITS-2 sequences gives high confidence that the W and J strain belong to the same species.

#### *Infection dynamics*

Parasitism is a lifestyle defined by the intricately-linked interplay between parasite and host. Knowledge of the infection dynamics of a parasite helps to decipher the host

response to infection, and build models for possible methods of intervention and vaccination. Several parameters which define infection dynamics are the parasite's infectivity, its survival in the host, and its capability to reproduce. In the current study, the W strain is representative of laboratory passaged *N. brasiliensis*, and the J strain is a recent field isolate, which is a better representative of natural infection. In terms of infection dynamics, the J strain was shown to have higher survivorship and fecundity. The two strains did not differ in infectivity or establishment, which suggests that the differences in survivorship and fecundity observed were not directly under the influence of density-dependent effects during the infection, as worm burden in the gut was the same for both strains at these time-points. Host response was also determined to be similar in type and magnitude towards both strains, and thus survivorship and fecundity are likely to be properties intrinsic to the parasite, probably manifested at the adult stage of *N. brasiliensis* and selected by previous life history conditions.

Although there has been limited data in the literature on the effect of routine laboratory passage on the life history traits of parasitic nematodes, a difference in survivorship has also been found in a study comparing two sibling lines of *T. muris* which were originally derived from the same stock but maintained in separate laboratories for approximately 100 generations in 30 years, even though their methods of life cycle maintenance were mostly similar (Bellaby *et al.*, 1995). Moreover, the authors also showed that a field isolate, more recently obtained from a wild mouse, can consistently survive for longer than the laboratory-passaged strains in various types of mice, although fecundity of the strains was not addressed. These results suggest that the survivorship of a parasite strain may be compromised after prolonged laboratory passage, similar to the situation with the W and J strain of *N. brasiliensis*.

Unlike the *N. brasiliensis* strains however, the *T. muris* strains were found to elicit differential immune responses in the host (Johnston *et al.*, 2005). Mice infected with the field isolate of *T. muris* experienced a profoundly downregulated Th2 response and retained the worms, whereas infection with the laboratory-passaged strain induced a strong and protective Th2 response which expelled the parasites. The higher survival of the *T. muris* field strain was therefore most likely due to its greater capacity for immunoregulation, which limited IL-4 and IL-13 associated goblet cell hyperplasia, mastocytosis and eosinophilia, subsequently evading immune expulsion.

In contrast, the mode and magnitude of host immune response to the *N. brasiliensis* strains were largely similar, suggesting that the differences in infection dynamics may not be attributed to these factors.

Although the decreases in survivorship and fecundity in the W strain are most likely to have been shaped by regimes of laboratory passage (reasons discussed in the next sub-section), there is also the possibility that they were legacies from their life history origins in different geographical locations. This limitation also applies to the studies on the *T. muris* strains, in which the laboratory passaged strains originated from Edinburgh and the field isolate was obtained in Portugal (Bellaby *et al.*, 1995). Propagation of a species in different geographical locations is likely to result in differences in life history traits, adapted to be most favourable in its particular environment, especially in light of the high turnover rate of generations and trait plasticity of nematodes. Studies on different geographical isolates of *T. spiralis*, a widely distributed parasitic nematode with a very promiscuous host range, provide an extreme example, exhibiting differential infectivity, immunogenicity, pathogenicity, antigenicity, and variations in repetitive DNA sequences (Bolas-Fernandez and Wakelin, 1990; Goyal and Wakelin, 1993; Wakelin and Goyal, 1996). With consideration to this, the infection dynamics of the J strain were re-assessed in 2010 after three years of laboratory passage and compared with data from 2007 when the strain was newly isolated. The finding that adult fecundity decreased significantly over 3 years is a strong indication that laboratory passage is a key influencing factor, although survivorship was not affected. Experimental selection of *S. ratti* larvae produced early in infection, which should in theory remove the need for parasites to invest in being long lived, also did not affect parasite survivorship but resulted in a change in fecundity of the parasite strains, suggesting that fecundity is perhaps a trait with higher plasticity (Paterson and Barber, 2007). It may perhaps require a greater number of laboratory passages for the effects on survivorship to become apparent.

### ***Laboratory infection regimes versus natural infections***

There are several ways in which laboratory infection regimes suffer a degree of artificiality, which are discussed below with reference to how repeated parasite

passage under these regimes may select for certain traits and affect infection kinetics, and the implications for natural infection.

### *Needle injection*

In natural infections with *N. brasiliensis* or other strongylid nematodes, most hosts are lightly infected with low numbers of infective larvae in the field through skin penetration (Scott and Lewis, 1987). In contrast, laboratory infections are typically carried out with a dose of several thousand infective larvae, directly injected into each animal subcutaneously. Infecting animals by injection bypasses the need for larvae to sense, penetrate and migrate through host skin, and injection with such large numbers may possibly allow parasites which are not as adept in the invasive process to slip by. These procedures pose little selection of fitness on the parasite, as host barriers have been artificially relaxed. Through prolonged laboratory passage, such factors which should regulate infectivity in the natural scenario may become redundant and lost due to a lack of selective pressure during propagation. Although infectivity was determined to be similar between the laboratory passaged and field strains in my experiments, it was measured by quantifying the worm numbers on days 3 and 4 post-infection via needle injection, which did not take into account the initial events of host invasion such as host-recognition and larval penetration. Thus while it can be concluded that laboratory passage did not affect the ability of the parasite to colonise the host, the ability to respond and penetrate the host was not compared. As an alternative to needle injection, it has been demonstrated that infections of *Strongyloides ratti* or *N. brasiliensis* can be established by direct application of small numbers of larvae onto the naked skin of rats (Tindall and Wilson, 1990a, b), but this method has not been widely utilised for *N. brasiliensis*.

### *Large infecting dose*

The infecting dose may have a direct effect on infection outcome and the host immune response during primary infections. It has been shown in the literature and my experiments that 'large single infections' (generally involving several thousand infective larvae) of *N. brasiliensis* result in a parasitic phase which lasts for 10-14 days, at the end of which adult worms are expelled by host immune mechanisms (Ogilvie and Jones, 1971). However, with small infections (50 L3), worm loss becomes gradual over a 30-day period (Haley and Parker, 1961). Moreover, when rats

are infected by daily exposure to small numbers of *N. brasiliensis* larvae (5 per day for 5 days a week), the infection profile is vastly different. These ‘trickle infections’ result in a gradual increase in worm burden which can be sustained for over 12 weeks. In addition, the worm population remained steady in the host a month after the administration of infective parasites is terminated, displaying the nature of chronic infections which is not seen in large single infections (Jenkins and Phillipson, 1971). It has been proposed, in the *T. muris* model, that chronic infections of parasitic nematodes can establish in the host under ‘trickle’ regimes because they do not trigger a Th2 response which is crucial for worm expulsion, instead biasing CD4<sup>+</sup> T cells to mature in the Th1 pathway (Bancroft *et al.*, 1994). However, in *N. brasiliensis* it was found that a Th2 response was still dominant in mice infected via a ‘trickle’ regime (5 infective larvae for 3 times a week), and that the infection was sustained for 3 months, at the end which worms were expelled, presumably because the threshold worm burden was reached (Selkirk, unpublished data). These data suggest that the infecting dose could have an important effect on the outcome of an infection as well as the host immune response, and the ‘trickle’ regime is probably much closer to the situation in naturally occurring parasitic nematode infections.

Although the type of immune response to large and trickle infections of *N. brasiliensis* is similar, a large infecting dose elicits a higher level of immune response which acts accordingly to regulate the worm population (Paterson and Viney, 2002). Through prolonged high-dose laboratory passage, parasite survivorship is consistently limited by a ferocious host immune response which increases in intensity as the infection progresses - it was shown in my experiments that cellular and cytokine responses of MLN cells to the parasites were higher on day 10 post-infection compared to day 7. Thus investment in survival past the egg-production period may not be particularly advantageous in this context, as expulsion is virtually inevitable considering the strength of response, and any progeny produced will continue to be artificially propagated in large amounts in any case. This could be a potential reason why the J strain can persist for longer in the host compared to the W strain. Similarly, even though investment in fecundity would increase the chances of propagation, the selective pressure presented by high-dose infections is probably much more relaxed compared to the natural scenario.



Alternatively, it is possible that survivorship and fecundity in the laboratory-passaged parasites were compromised in a trade-off with other life history traits which would be more useful in aiding propagation of the species in the long run (Paterson and Barber, 2007). The effect of such trade-offs may not be immediately apparent, since the interactions between selective forces can be very complex. It would be tempting to suggest that survival and/or fecundity of the W strain was decreased under a trade-off with age to maturity to enable an accelerated phase of egg production, the latter of which would make particular sense since investment in a shorter time to maturation would conceivably result in a smaller worm size, which limits fecundity (Poulin, 1998). This theory has been supported by an optimality model for maturation time based on the fecundity, mortality rate and prepatent period of 28 gastrointestinal nematode species (Gemmill *et al.*, 1999). It was observed in my experiments that egg production of the W strain initiated and peaked slightly earlier than the J strain, even though results did not reach statistical significance, but comparison with data in the literature do seem to suggest that the egg production period of the W strain has shifted forward (Ball, 2004; Kassai, 1982; Ogilvie and Jones, 1971). However, this effect was not replicated in the J strain after three years of laboratory passage.

Another feature of the study by Paterson and Barber (2007) suggests that parasitic traits may undergo selection by repeated passage of parasite progeny produced early or late in infection. Thus routine laboratory passage may potentially select for sub-populations of worms if larvae from eggs produced very early or very late in infection was repeatedly used for life cycle maintenance. In consideration to this, larvae from eggs produced mid-cycle or a mixture from all days post-infection has been used for infections to avoid this potential consequence.

#### *Host homogeneity*

Lastly, the life cycle of *N. brasiliensis* is typically maintained by serial passage in laboratory rats, usually making use of a single host strain. In our laboratory, outbred male Sprague Dawley (SD) rats were used for all passages and experimental infections. However, as was shown in the serial passage of *H. polygyrus* in Quackenbush mice, host homogeneity can restrict infection of the parasite to a particular host strain (Dobson and Owen, 1977), whereas in nature its host strain

preference may be more relaxed. Serial passage of *N. brasiliensis* in SD rats could thus potentially restrict its diversity in infection of different rat strains.

### ***Epidemiological perspectives in the use of laboratory passaged parasite strains***

It has been a concern of many parasitologists that laboratory infections may have limited relevance for natural infections, and that a laboratory-passaged strain would not be able to correctly represent the parasite in the wild (Maizels and Kurniawan-Atmadja, 2002; Paterson and Viney, 2002). In the current study, I have found that the infectivity (at least after the events of skin penetration) and establishment of the parasite was not affected by laboratory passage. The host immune response was also similar, so that immunological studies on laboratory-passaged parasites should still be relevant. However, the laboratory-passaged strain displayed a decrease in survivorship and fecundity, which would result in incorrect parameters for parasite clearance and transmissibility in epidemiological predictions in the natural setting. Virulence of the parasite, which is defined as the degree of pathogen-induced host mortality, usually assessed by LD50 (lethal dose which results in 50% of death in the host population), was not experimentally assessed due to ethical reasons. However, both strains were found to cause death of the host when infected with over 10,000 L3, so that the upper limit of worm dose (and thus perhaps virulence) appears to be similar in the two strains. Considering that multiple parameters which define infection dynamics can be affected by prolonged laboratory passage, the importance of field studies in advising epidemiological models is highlighted.

### ***Secreted proteins***

The W and J strain secrete similar amounts of proteins during both the L3A and adult stage, but their secreted protein profiles show some subtle differences. This may suggest that the two strains have faced different objectives in relation to their allocation of resources under their different life history conditions, traits which may have co-evolved with their secreted proteins. This is not surprising, as secreted proteins are the primary interface between the parasite and host. As discussed

regarding the limited selection of fitness in laboratory passage regimes, proteins which may otherwise be of high importance in natural infections may become redundant and gradually eliminated from the secretome of the W strain. Within the context of this study, the comparison of adult secreted proteins between the strains is probably more relevant to parasite survivorship and fecundity.

Since the production of proteins is ultimately determined by genetic information, it is intuitive to assume that genetic variation would have an underlying basis in the adaptation of parasite life history traits, in which those with genotypes which can thrive in a particular environment will be selected and propagated. A clue to this would be that the adaptations acquired are inheritable and passed on through generations of progeny. For example, in adapting *N. brasiliensis* to mice, infectivity gradually rose from 14% to 56% over four passages, and furthermore, the resulting strain showed a decreased ability to infect rats (the original host) after eight passages (Wescott and Todd, 1966). Similarly, passaging *Schistosoma mattheei* through hamsters lowered its pathogenicity for sheep, the natural host (Taylor *et al.*, 1977). In terms of molecular evidence, *Strongyloides ratti* lines with different genotypes were found to vary in survivorship and fecundity during infections (Paterson and Viney, 2003). A more specific example of genetic alterations underlying parasite adaptation comes from experiments in which an African isolate of *Schistosoma mansoni* was maintained separately for twelve years in baboons and mice. The baboon line of *S. mansoni* showed polymorphic alleles at several loci, whereas single alleles were fixed in the mouse line at the same loci. After serial passage of the baboon line through laboratory mice for four generations, the resulting progeny were found to have fixed the same alleles as the original mouse strain, providing strong molecular evidence for host-induced selection (LoVerde *et al.*, 1985).

It is not surprising that immune sera to the W and J strain can cross-react with secreted proteins of each other in the immunoblotting experiment, as their secretion profiles are largely similar. On the other hand, the fact that strain-specific reaction was stronger suggests that the secreted proteins of the two strains are not completely identical. In addition, it was interesting that J strain-specific IgG is capable of higher recognition of proteins secreted by both the W and J strain than that of the W strain-specific IgG. Although peripheral antibody responses are generally not related to

primary expulsion kinetics (Else and Grecnis, 1996; Jacobson *et al.*, 1977), it has been shown in *S. ratti* that parasite survivorship is negatively correlated to parasite-specific IgG1 and IgA, whereas fecundity and IgA levels are inversely correlated (Bleay *et al.*, 2007). The case for antibody responses influencing the outcome of *N. brasiliensis* infection however, is unclear but unlikely to be of high importance to immunity (Jacobson *et al.*, 1977; Liu *et al.*, 2010).

In terms of secreted enzymes, it was determined that secreted proteins of the J strain did not display higher levels of AChE and nucleotide metabolising enzyme activities, suggesting that they are not directly responsible for the difference in persistence and fecundity between the strains. Although the secretion of AChE has been well described in *N. brasiliensis* (Hussein *et al.*, 2002), this report provides the first data showing that this parasite secretes nucleotide metabolising enzymes also. The specific nucleotide metabolising activities in *N. brasiliensis* secreted proteins appear to be much higher than that of *T. spiralis* (Gounaris, 2002). However, these conclusions should be qualified by the fact that total secreted proteins may contain multiple enzyme activities which complicate analysis, and it would therefore be interesting to identify the enzymes responsible for nucleotide hydrolysis in *N. brasiliensis* before direct comparison with those of other nematodes. Secondly, the substrate specificities of the nucleotide metabolising enzymes of *N. brasiliensis* appeared to differ to those of *T. spiralis*, and more akin to the apyrases secreted by arthropods (Gounaris and Selkirk, 2005). It was recently discovered however, that *T. circumcincta* and *O. ostertagi* also secrete an enzyme apyrase which hydrolyses ATP, ADP, but not AMP (Nisbet *et al.*, 2011; Zarlenga *et al.*, 2011), the substrate specificities of which are closer to that of *N. brasiliensis* than to *T. spiralis*. Apart from indicating that the degradation of host nucleotides could be a common nematode strategy in immunoevasion, this may also suggest that the enzymatic schemes responsible for this phenomenon may differ between nematode clades.

## *Chapter conclusions*

In the current study, strong data has been presented to suggest that laboratory passage of parasitic nematodes such as *N. brasiliensis* could have profound effects on infection dynamics, which may ultimately misrepresent events of a natural infection, bias epidemiological models and potentially compromise efficacy of drugs and vaccines. Although it is not clear as to why the survivorship and fecundity of *N. brasiliensis* decreased after prolonged laboratory passage, it is most likely that 1) large infecting doses by needle injection relaxes the need for selection of the fittest, and 2) a possible trade-off of survival and fecundity may have occurred with an unknown trait that is not immediately apparent but is favourable in the laboratory-passage context. Moreover, although a direct cause for the differences in infection dynamics could not be inferred through studies on host response and enzyme activities, this is likely to be determined by a plethora of biological activities rather than one or two major factors which could be measured by the assays employed here. Nevertheless this study is a cautionary tale to the potential dangers of practising multiple passages of parasites. By relaxing natural constraints to ensure a successful infection every time (i.e. infecting with large numbers of parasites and direct injection), we are repeatedly subjecting the parasites to a process in which infection is made more facile. This may make redundant functions important in a natural infection, along with the proteins responsible for them. As multiple passage of parasitic nematodes cannot be avoided in laboratory use, it may be advantageous to do this at the minimal infection dose possible, avoid selecting for sub-populations of worms, and explore methods of worm administration other than needle injection.

# **Chapter 4**

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## **Activation and protein secretion of infective larvae**

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## 4.1 Introduction

The initial events of the infectious process in parasitism are major determinants of the eventual infection outcome. The establishment of an infection is often dependent upon the parasite's ability to locate, recognise, invade, colonise and develop in its host, which requires its infective stage to sense and respond to host signals appropriately. At its free-living, infective stage, many parasitic nematodes are developmentally arrested in an environmentally resistant state of metabolic repression, non-feeding with low motility (Ogilvie and Jones, 1971), which lowers the energy cost during the waiting period. Host factors activate the infective larvae to resume development and enter into its parasitic phase, an event known as the 'transition to parasitism', or 'activation' (Hawdon *et al.*, 1992). Once the parasite has entered its host, the factors for nematode activation become a black box to researchers. However, by breaking down the separate factors in the host environment individually, it can be studied in components by *in vitro* methods.

It is evolutionarily feasible for a parasite to optimise its chances of host encounter and invasion to ensure its successful transmission and propagation. Most parasitic nematodes produce infective larvae in large amounts, which position themselves to maximise host encounter. The infective larvae of most soil-transmitted strongylid nematodes, including those of *N. brasiliensis*, are known to exhibit negative geotropism in which they crawl up objects, such as the tips of grass stems, for maximal likelihood of host contact (Haas, 2003; Kassai, 1982; Smyth, 1988). Once a host is encountered, the parasite has a small window of opportunity for invasion, within which it would need to sense and react to host signals appropriately. Many parasitic nematodes have been known to be responsive to chemical and thermal cues. Skin-penetrating larvae of *A. caninum* and *Strongyloides stercoralis* are attracted to the hydrophilic (aqueous) fraction of host skin (Granzer and Haas, 1991), and in the latter it was found that urocanic acid, a generic chemical found in most mammalian skin which is particularly concentrated in the foot (the mostly likely site of larval penetration), was the major chemoattractant (Safer *et al.*, 2007). Infective larvae of most parasitic nematodes which infect warm-blooded animals are also positively thermotactic, suggesting that temperature could be another important factor in host finding and invasion (Granzer and Haas, 1991; Haas, 2003; Smyth, 1988).

During host invasion, infective larvae are activated by host factors to transit into their parasitic stage. At this point a string of events are triggered, including exsheathment of the outer cuticle (retained from the previous L2 stage), secretion of proteins, resumption of feeding, and reinitiation of the developmental program (Hawdon and Hotez, 1996). Resumption of feeding is frequently used as a marker for activation, because it is a distinguisher which can be easily sampled *in vitro* by feeding worms with fluorescent protein. Using this method, the factors which trigger activation of *A. caninum* L3 have been extensively studied. At host-like temperatures, feeding in *A. caninum* can be stimulated by exposure to canine serum and reduced glutathione (GSH), independent of pH and culture medium (Hawdon and Schad, 1990, 1992, 1993). Serum and GSH can individually induce feeding, but show a synergistic effect when combined. Both compounds display concentration-dependent effects on feeding activation up to 10% for serum and 50 mM for GSH, at which saturation effects become apparent. Once exposed to these factors for a short period, the pathway to feeding can be activated without the continued presence of these stimuli, indicating a trigger / switch mechanism for activation. The trigger and saturation effects suggest that activation is a receptor-mediated signalling process. However, the trigger signals for activation may differ between nematode species. In a study of hookworm activation, serum and GSH were found to stimulate feeding in larvae of various species within the genus *Ancylostoma* to varying extents, but had no effect on *Necator americanus* (Hawdon *et al.*, 1992), findings which cautions against over-generalisations in the study of hookworm biology.

The developmentally arrested L3 stage of parasitic nematodes is often compared to the dauer (German for resting, enduring) stage of *C. elegans*, which are phenotypically and functionally similar (Hotez *et al.*, 1993). Under conditions of low food supply, high pheromone concentration (indicator of overcrowding) or high temperatures, L1 larvae of *C. elegans* may choose to enter an alternate state of dormancy, referred to as the dauer stage, after the second larval moult (Cassada and Russell, 1975; Golden and Riddle, 1984). Like the L3 stage of parasitic nematodes, the dauer stage is metabolically and developmentally repressed, non-feeding, ensheathed in a tough cuticle and resistant to harsh environmental conditions. *C. elegans* larvae can remain viable for many months in its dauer form, until favourable



environmental conditions induce them to exit the dauer stage and resume development to become reproductive adults. Indeed, this mechanism exhibits striking parallels with the transition of free-living L3 into its parasitic stages upon host entry, which encouraged many investigators to equate the hookworm activation process with dauer recovery.

During invasion, larvae of parasitic nematodes are also activated to secrete a plethora of proteins, which may be involved with the penetration of defensive barriers, the avoidance or modulation of host immune response, the facilitation of feeding, survival and host colonisation (Hawdon and Hotez, 1996). These generally include a range of proteases, protease inhibitors, hydrolytic enzymes, cytokine homologues and antioxidant enzymes (Dzik, 2006). Very little is known about the specifics of how they facilitate the invasion process, but a few invasion factors have been studied. Notably, activated larvae of *A. caninum*, *Ancylostoma brasiliensis*, *Anisakis simplex* and *H. contortus* secrete an active hyaluronidase which is likely to facilitate tissue degradation, thus enabling passage of larvae through the epidermis and dermis during migration (Hotez *et al.*, 1994; Hotez *et al.*, 1992; Rhoads *et al.*, 2000). Metalloproteases such as MTP-1 secreted by *A. caninum* L3 upon serum-stimulated activation showed an ability to digest tissue substrates and facilitate larval migration *in vitro* (Williamson *et al.*, 2006; Zhan *et al.*, 2002). Ensheathed larvae have also been reported to secrete an 'exsheathing fluid', which includes collagenases, proteases and lipases, which are able to digest the tough cuticles that are normally resistant to host and environmental attack (Rogers, 1982; Sommerville and Rogers, 1987). A more recent study has investigated the transcriptional changes in *A. caninum* during activation. It was found that the gene set which was upregulated in activated L3 differs substantially from that of *C. elegans* during dauer recovery (Datu *et al.*, 2008), suggesting that they may represent unique parasite-specific factors. A large number of the upregulated genes were predicted to be extracellular (likely to be secreted), with putative roles in host-parasite interactions. These include a range of cysteine-, aspartyl- and metalloproteases, as well as an enzyme apyrase and multiple members of the VAL protein family.

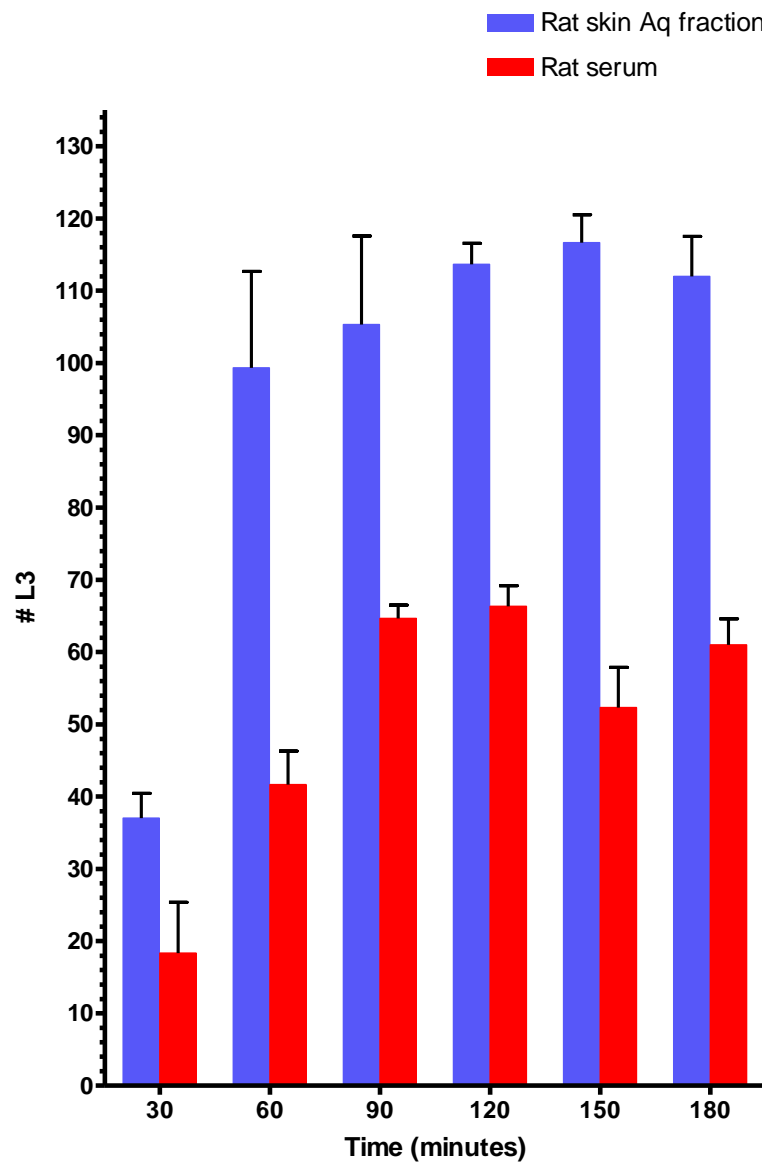
The objective of this chapter is to investigate the factors which influence the activation and protein secretion of *N. brasiliensis* L3. Host-like factors which have

been successful in triggering activation in other nematode species were mimicked in my experiments, and the outcome assessed using an *in vitro* feeding assay. Chemotaxis assays were also carried out to discover substances to which *N. brasiliensis* L3 are responsive, and promising candidates were then tested as stimulants for activation. The influence of these factors on protein secretion of L3 was then investigated qualitatively through the use of one- and two-dimensional gel separation methods. Finally, the effect of resistin-like molecules (RELMs) on the early events of activation was studied, as a member of this protein family, RELM $\beta$ , has been found to inhibit larval chemotaxis and adult feeding in the parasitic nematodes *S. stercoralis* and *H. polygyrus* respectively (Artis *et al.*, 2004; Herbert *et al.*, 2009).

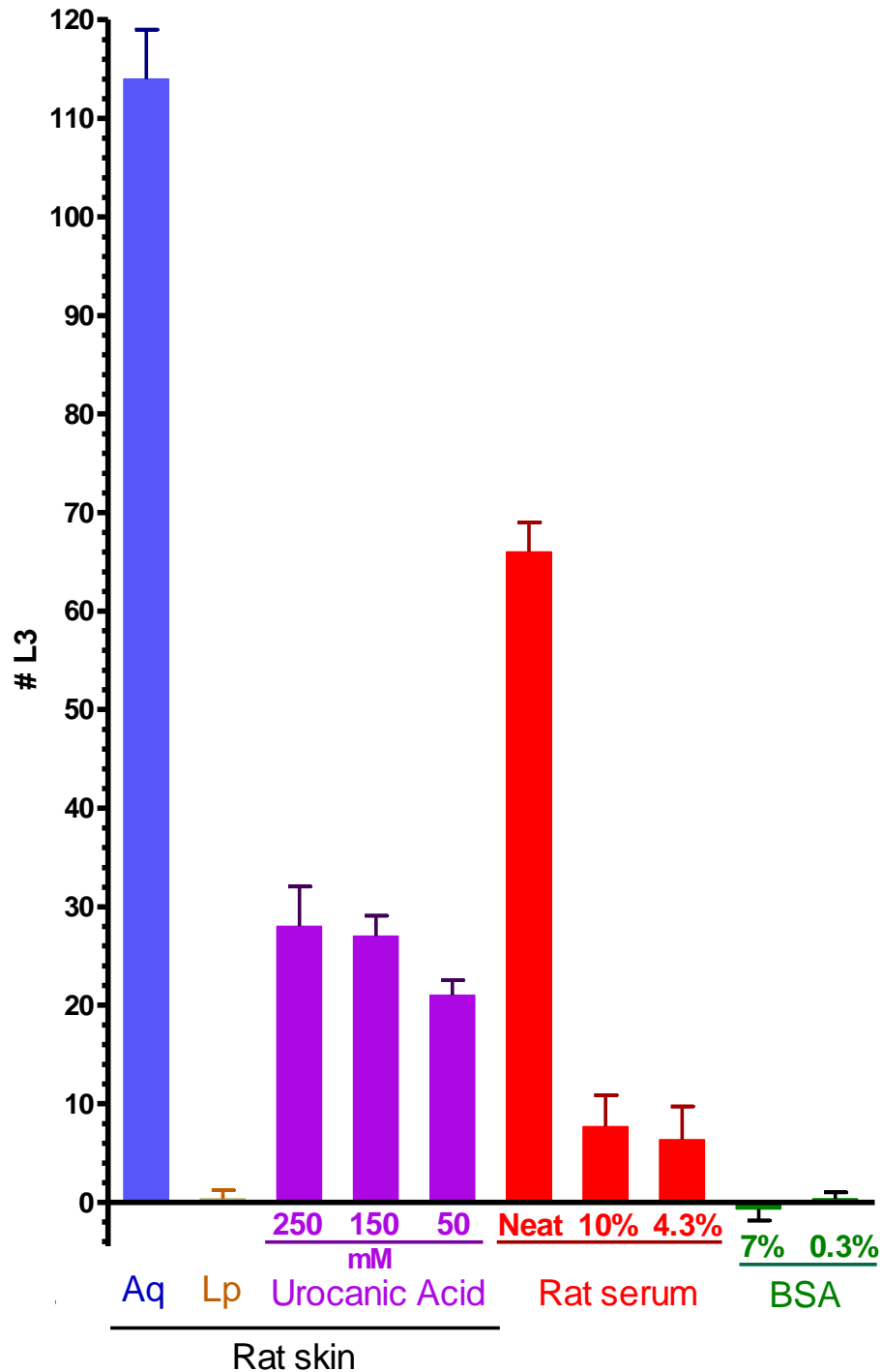
## 4.2. Chemotaxis of *N. brasiliensis* infective larvae

Chemotaxis assays were carried out to elucidate the chemical cues which attract *N. brasiliensis* L3. This was done by monitoring larval migration on solid agarose, with test wells punched into opposing edges of the plate. When only water was added into the test wells, L3 migrated randomly or remained stationary. The aqueous fraction (Aq) of rat skin and rat serum were then tested as chemoattractants in a time-course experiment (Fig. 4.1). Directed migration of L3 towards both chemoattractants were observed by 30 minutes after the addition of worms and increased over time, until the numbers were stabilised by 120 minutes into the experiment. At every time-point sampled, the aqueous fraction of rat skin proved to be a stronger chemoattractant than rat serum. A comparison of other substances as chemoattractants were carried out at the 120-minute time point (Fig. 4.2), in which L3 were found to be most attracted to the aqueous fraction of rat skin. The lipid fraction (Lp) of rat skin had no effect on the migration of *N. brasiliensis* L3, but the worms showed some directed chemotaxis towards urocanic acid, albeit to a lesser extent than to the aqueous fraction of rat skin. *N. brasiliensis* L3 were moderately attracted to rat serum, and the degree/level of chemoattraction diminished as serum concentration was decreased. Migration of L3 was not affected by 0.3% or 7% BSA (equivalent protein concentration to the aqueous fraction of rat skin and neat rat serum respectively), showing that the worms were not solely migrating up a generic protein gradient, but the process is dependent on specific chemical cues derived from the host.

The chemotaxis assays were originally carried out on 0.5% and 0.7% agar plates, as suggested by experiments on *S. stercoralis* (Safer *et al.*, 2007). Interestingly, a number of *N. brasiliensis* L3 penetrated into the soft agar equilibrated with the test substances, evident when the aqueous fraction of rat skin or rat serum were used as chemoattractants. This suggests that these substances may have induced penetrative behaviour in *N. brasiliensis* L3. However, the larvae which penetrated often got stuck within the agar, which made the scoring of positive chemotaxis in the test wells difficult and inconsistent. The assay was then modified to use 0.9% agarose as the solid medium, the surface on which the larvae could glide freely but did not penetrate, so that the distinction of directed chemotaxis towards different chemical cues could be determined more uniformly and reliably.



**Figure 4.1. *N. brasiliensis* L3 worms show positive chemotaxis towards the aqueous (Aq) fraction of rat skin and rat serum.** Results show the time course of chemotaxis expressed as mean numbers of L3 in the positive well (containing test chemoattractant, i.e. rat skin aq fraction or rat serum) less those in the negative well (containing water) + SEM (where  $n = 3$ ), assayed in triplicate. The assays were carried out on 0.9% agarose plates. Numbers were scored at the indicated time points after the addition of L3 (2 droplets each of 150 worms) onto the test plates.



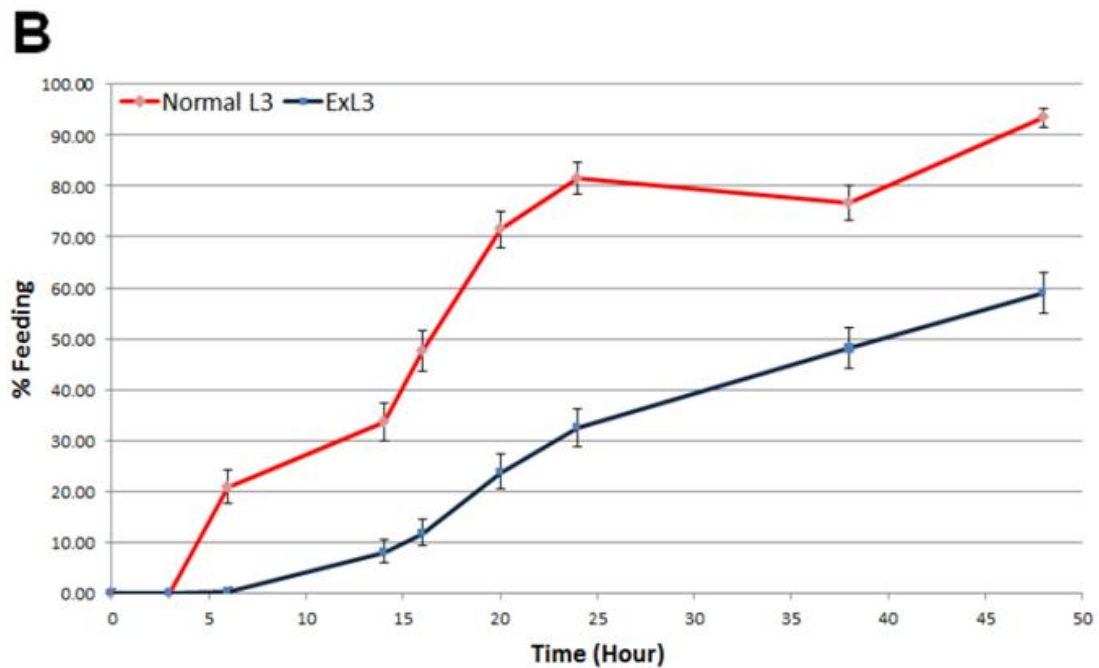
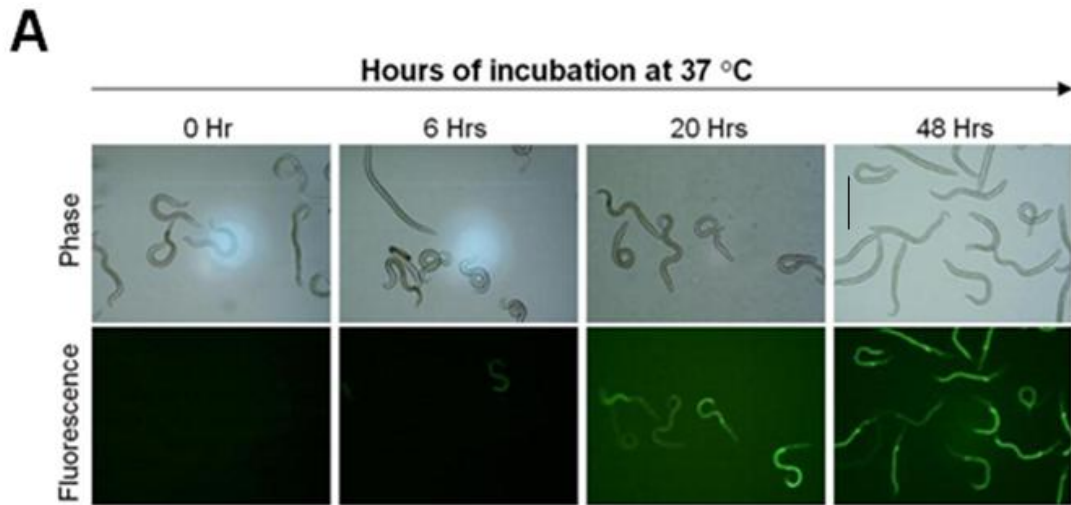
**Figure 4.2. Chemotaxis of L3 towards constituents of rat skin and rat serum.** Results show the time course of chemotaxis expressed as mean numbers of L3 in the positive well (containing test chemoattractant) less those in the negative well (containing water) + SEM (where  $n = 3$ ), assayed in triplicate. Numbers were scored 120 minutes after the addition of L3 (2 droplets each of 150 worms) onto the test plates. Aq: aqueous fraction; Lp, lipid fraction; BSA; bovine serum albumin. The assays were carried out on 0.9% agarose plates.

### 4.3. Activation of feeding in *N. brasiliensis* L3

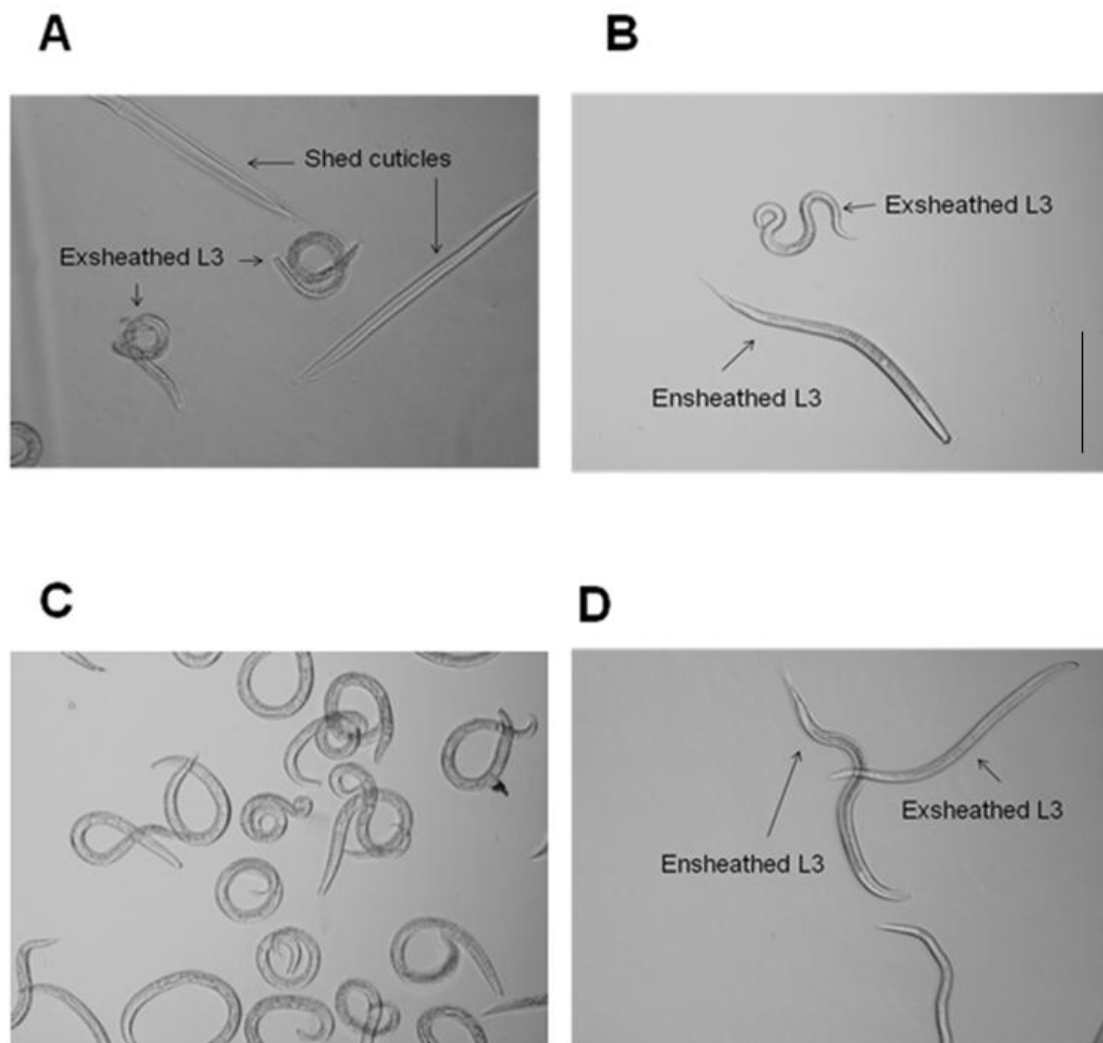
Initiation of feeding is a hallmark of larval activation often used to determine the emergence of L3 from its state of developmental arrest, as it is a distinguisher which can be sampled relatively easily by measuring the worms' ingestion of fluorescent FITC-BSA. Initiation of feeding in normal L3 was induced from 6 hours after cultivation at 37°C, as evident from fluorescence in the worm's alimentary tract (Fig. 4.3A), and the percentage of feeding worms increased over time up to approximately 90% at the 48-hour time point (Fig. 4.3B).

Exsheathment is a prerequisite for feeding, as the tough outer cuticle envelops the worm and obstructs its buccal opening. Freshly isolated larvae consist of a mixture of ensheathed and exsheathed worms, suggesting that exsheathment can occur naturally without an activation stimulus. When larvae were cultured at 37°C, worms were observed to exsheath, as evident by the shedding of their cuticles in the culture medium (Fig. 4.4A). Exsheathed larvae were smaller and tended to curl up during cultivation at 37°C (Fig. 4.4B). Feeding was observed in exsheathed larvae only, at all time-points and conditions sampled. By 24 hour cultivation at 37°C, all larvae were observed to be exsheathed (Fig. 4.4C). In contrast, the morphology of L3 cultured at 20°C remained the same, and no empty cuticles or feeding activities were observed in any of the experiments conducted at this temperature (Fig. 4.4D).

Exsheathment of worms can also be achieved chemically with the use of sodium hypochlorite. However, chemically exsheathed L3 actually showed a lower percentage of feeding activation than normal L3 at all time points, achieving only 50% feeding by 48-hour after cultivation at 37°C (Fig.4.3B). While it was possible that sodium hypochlorite treatment may have had harmful effects on the worms, it may also mean that activation of feeding cannot be achieved solely by the removal of the worm's outer cuticle. The experiments were also carried out at 20°C, and no feeding activity was observed in L3 with or without chemical exsheathment.



**Figure 4.3. Time course of feeding activation of *N. brasiliensis* L3 at 37°C.** Ingestion of FITC-BSA results in fluorescence in the alimentary tract of L3, indicating feeding activation. A) Images of L3 under phase and fluorescence microscopy at the indicated time points. Scale bar (black): 400  $\mu$ m. B) Percentage of feeding was sampled at 9 time points (0, 3, 6, 14, 16, 20, 24, 38 and 48 hours) from triplicate cultures (200 worms were sampled from each culture), expressed as mean values with the error bars representing the 95% confidence intervals (CI), in red for normal L3 and blue for chemically exsheathed L3 (ExsL3).

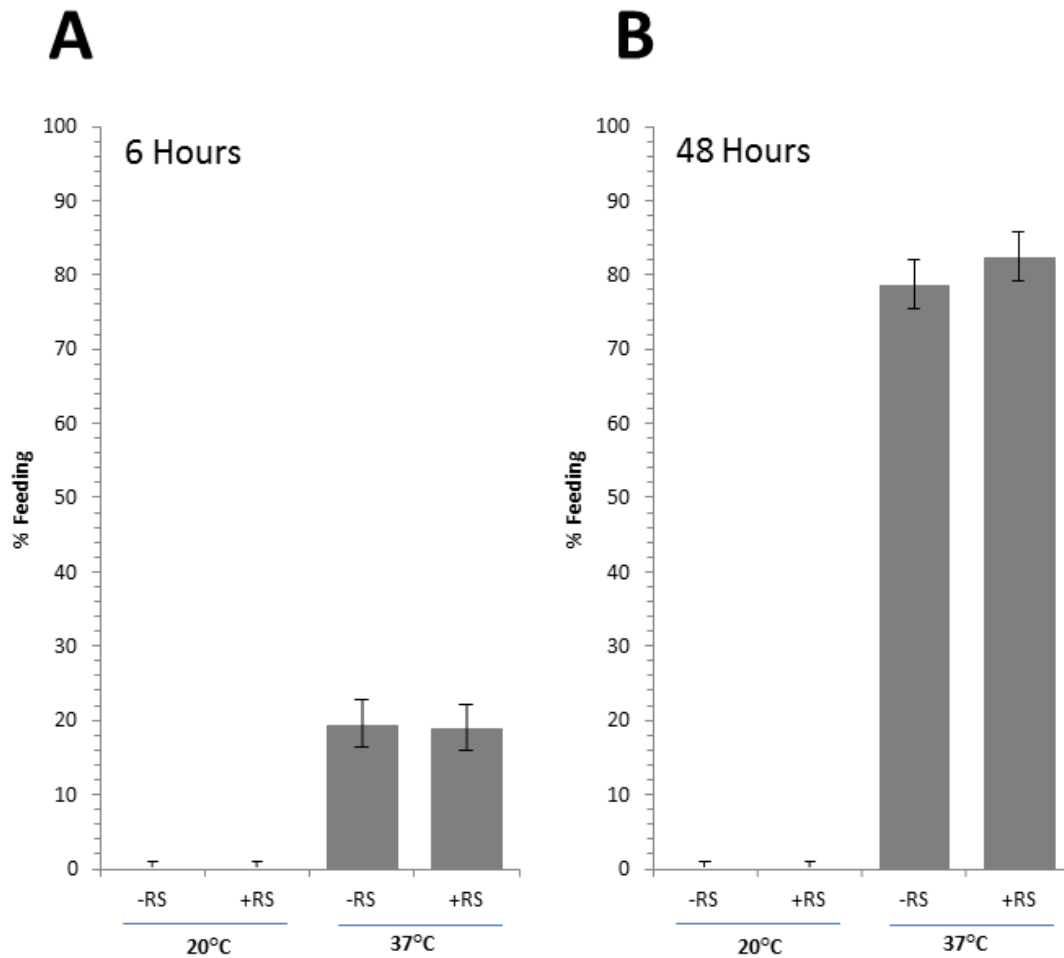


**Figure 4.4. Morphology of exsheathed and ensheathed L3 during cultivation.** All images photographed at x100 magnification. A) Morphology of exsheathed L3 at 37°C. Shed cuticles were also visible under light microscopy. B) Comparison of exsheathed and ensheathed L3 at 37°C. The former is enveloped in a tough cuticle, distinguished by a tapered posterior end. Both pictures (A and B) were taken at 6 hours post-activation at 37°C. C) All larvae were exsheathed by 24 hours activation at 37°C. D) Morphology of exsheathed and ensheathed L3 at 20°C, 24 hours into incubation. The ridged surface of ensheathed L3 can be observed in this picture. Scale bar (black): 400 μm.

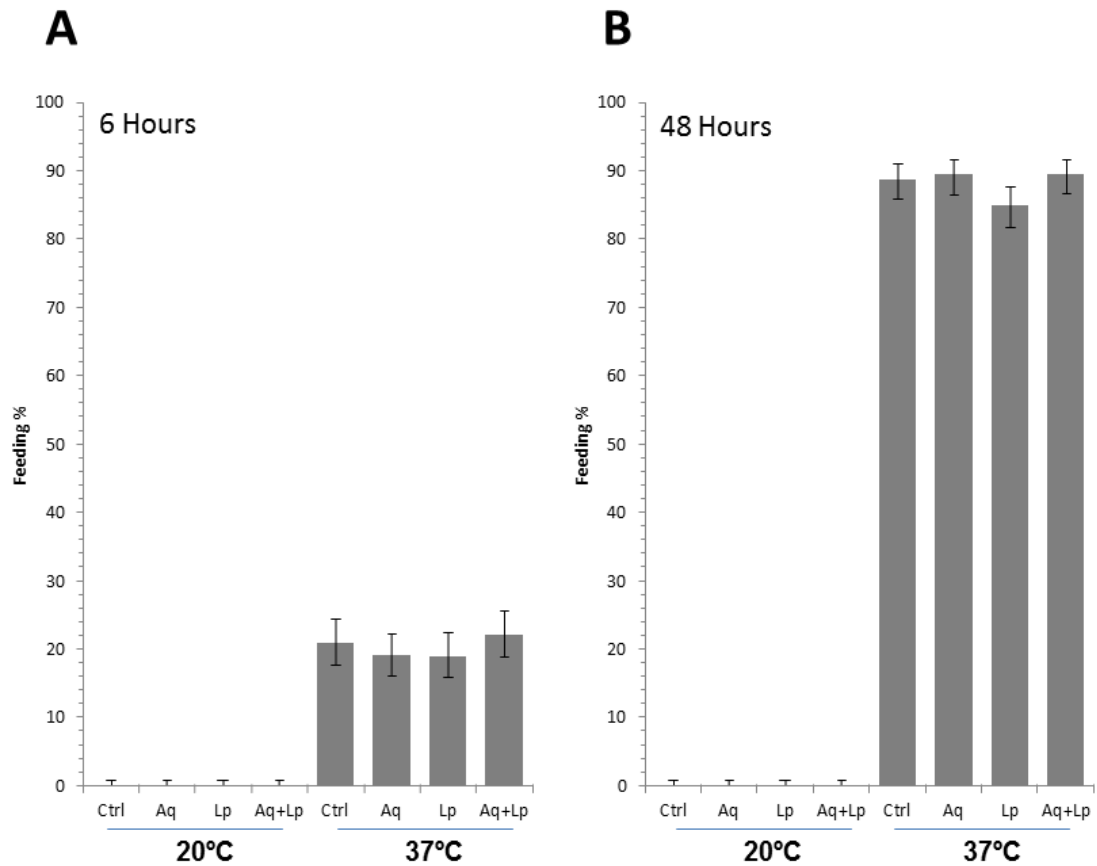


I next sought to investigate if rat serum had an effect on activation of feeding in *N. brasiliensis* L3. The experiments were carried out at 20°C and 37°C, with or without the inclusion of rat serum in the culture medium (Fig. 4.5). It was found that feeding activities cannot be initiated at 20°C, regardless of the presence of rat serum, suggesting that temperature is a critical factor for activation. Feeding was activated at 37°C by 6 hours into cultivation to approximately 20%, which increased to about 90% by 48 hours, and rat serum had no effect on the feeding percentage at either time point.

Similarly, assays were carried out to investigate the effect of rat skin fractions (aqueous and / or lipid) on feeding activation (Fig. 4.6). The results were similar to that of rat serum; neither the aqueous or lipid fraction or rat skin, or a combination of both, influenced feeding activation in *N. brasiliensis* L3 at 20°C or 37°C. In a separate experiment, a whole piece of rat skin was included in the culture, and feeding was also unaffected.



**Figure 4.5. The effect of temperature and rat serum (RS) on activation of feeding.** Where indicated (+RS), 10% rat serum was included within the medium in which *N. brasiliensis* L3 were cultured, at 20°C or 37°C. Feeding was assessed by ingestion of FITC-BSA and sampled at 6 or 48 hours from the beginning of the cultivation period in triplicate. Results are expressed as mean values with error bars representing the corresponding 95% confidence intervals (CI).



**Figure 4.6. The effect of temperature and rat skin on feeding activation.** Where indicated, the aqueous (Aq) or lipid (Lp) fraction of rat skin was included within the medium in which *N. brasiliensis* L3 were cultured, at 20°C or 37°C. No additives were added to the control (Ctrl) condition. Feeding was assessed by ingestion of FITC-BSA and sampled at 6 or 48 hours from the beginning of the cultivation period in triplicate. Results are expressed as mean values with error bars representing the corresponding 95% confidence intervals (CI).

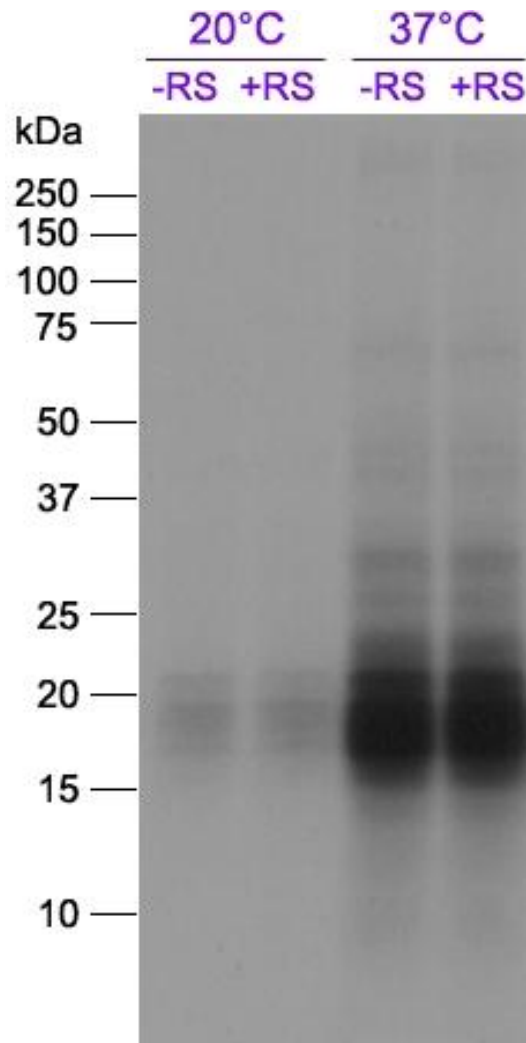
#### 4.4. Activation of larval protein secretion

The conditions which stimulate L3 to secrete proteins were investigated. The secreted proteins were metabolically radiolabelled by the addition of  $^{35}\text{S}$ -methionine ( $^{35}\text{S}$ -Met) in the medium with the worms during culture, to assess *de novo* protein synthesis under the specified conditions. At 20°C, L3 secrete only a small amount of proteins, whereas much greater amounts were secreted at 37°C (Fig. 4.7). Rat serum did not affect the secretion profile at either temperature. The labelled proteins secreted at 37°C were further resolved by two-dimensional gel electrophoresis (2-DE) for a finer comparison, in which the secretion profiles were again mostly indistinguishable in the presence or absence of rat serum (Fig. 4.8 and 4.9, secreted proteins of W strain and J strain worms respectively). L3 were then cultured in the same manner but with addition of rat skin fractions instead of rat serum. It was found that neither the aqueous or lipid fraction of rat skin affected the secretion profile of L3, at either 20°C or 37°C (Fig. 4.10).

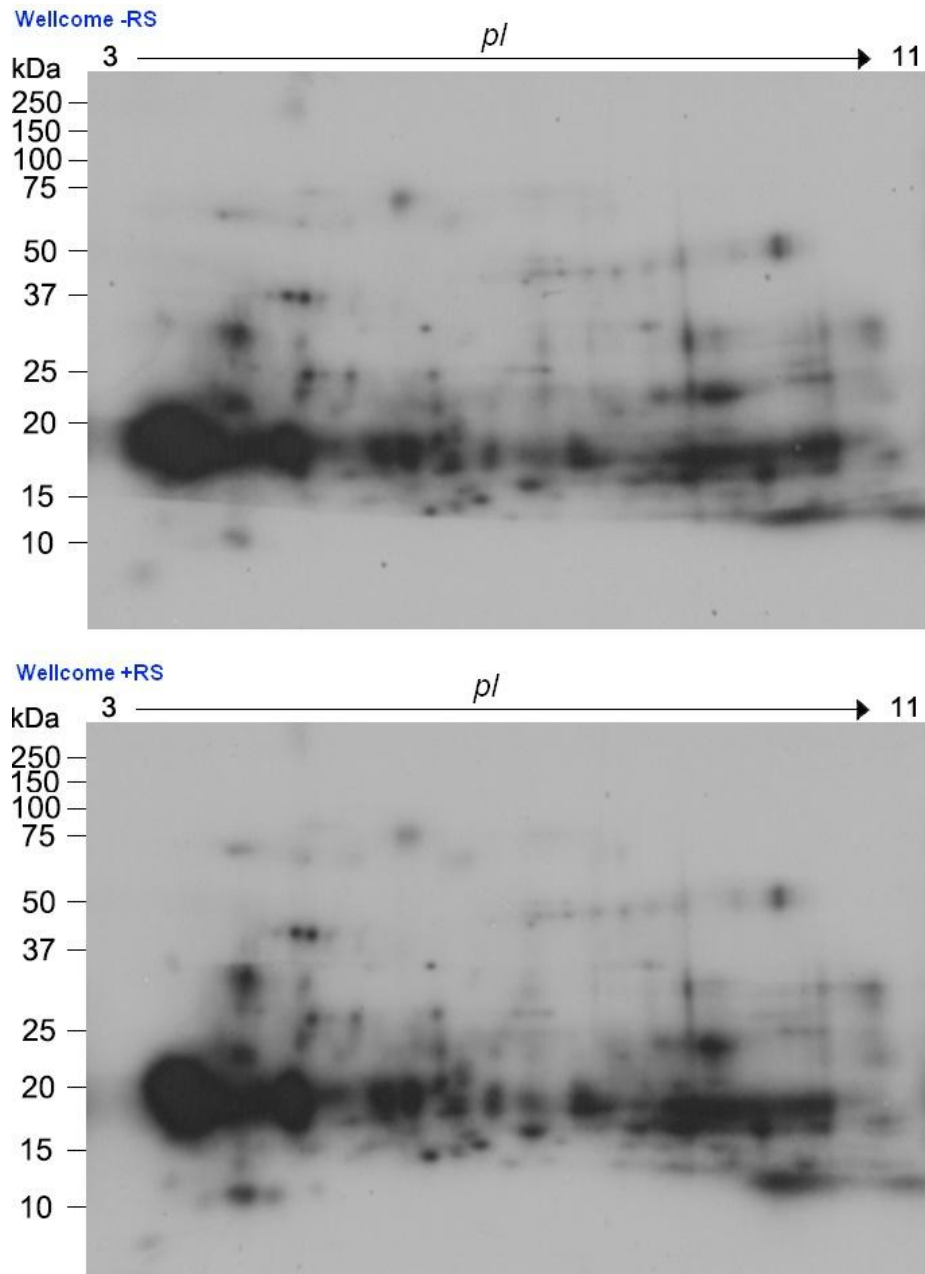
Apart from the activation of *de novo* protein synthesis, some parasitic helminths have also been known to pre-synthesise and store proteins during the pre-parasitic phase, which were then released upon activation. To investigate if such release of secreted proteins can be induced in *N. brasiliensis*, L3 were pre-labelled by incubation with  $^{35}\text{S}$ -Met at room temperature for 40 hours, washed extensively to remove the free excess radioisotope, then cultured for 30 minutes in fresh medium under the specified conditions, after which the proteins secreted during this short period were collected, resolved on SDS-PAGE, and visualised by autoradiography (Fig. 4.11). It was found that an elevation of temperature to 37°C alone did not induce release of pre-synthesised proteins. As the initial penetration of larvae into host skin presumably involves the secretion of hydrolytic enzymes, I also sought to investigate if exposure to rat skin fractions may stimulate the release of pre-synthesised proteins. The aqueous fraction of rat skin stimulated the release of a small bolus of proteins, to the same effect at 20°C and 37°C (Fig. 4.11). The lipid fraction of rat skin also stimulated this secretion but to a lesser extent.

Pre-synthesised proteins released by the aqueous fraction of rat skin were resolved on 2-DE (visualised by autoradiography) and compared with the normal secretion profile

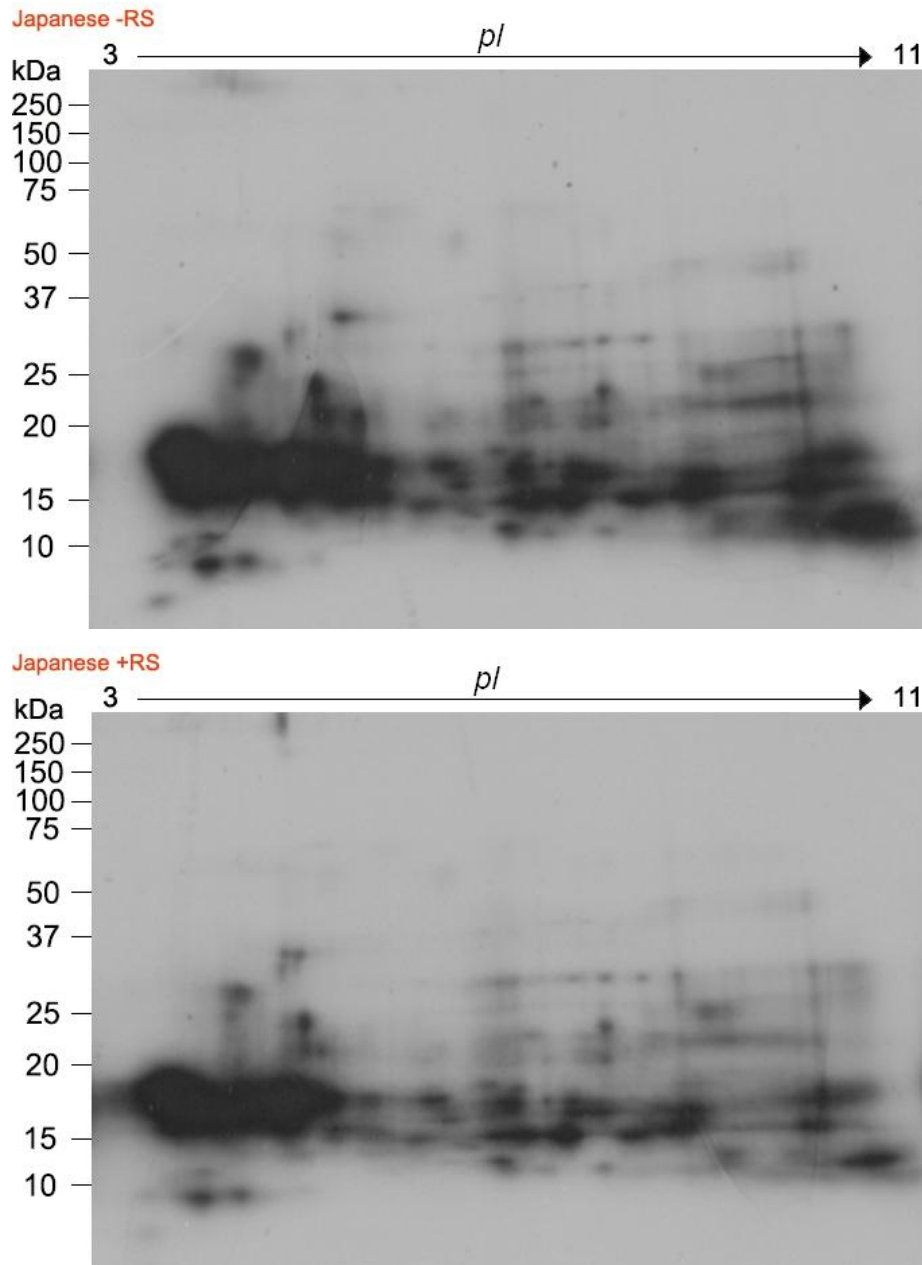
at 37°C (visualised by Coomassie blue staining). It was found that these pre-synthesised proteins were identical to those normally secreted at 37°C (Fig. 4.12), suggesting that there is no specific subset of proteins synthesised and released in this manner.



**Figure 4.7. Protein secretion by *N. brasiliensis* L3 is activated at 37°C, and is unaffected by exposure to rat serum (RS).** L3 secreted proteins (J strain) were metabolically labelled with  $^{35}\text{S}$  methionine ( $^{35}\text{S}$ -Met), resolved by 10-20% gradient SDS-PAGE and visualised by autoradiography. L3 were pre-sensitised with rat serum (+RS) where indicated. Each lane shows the protein secreted by 1700 L3 over a cultivation period of 2 days under the indicated conditions.

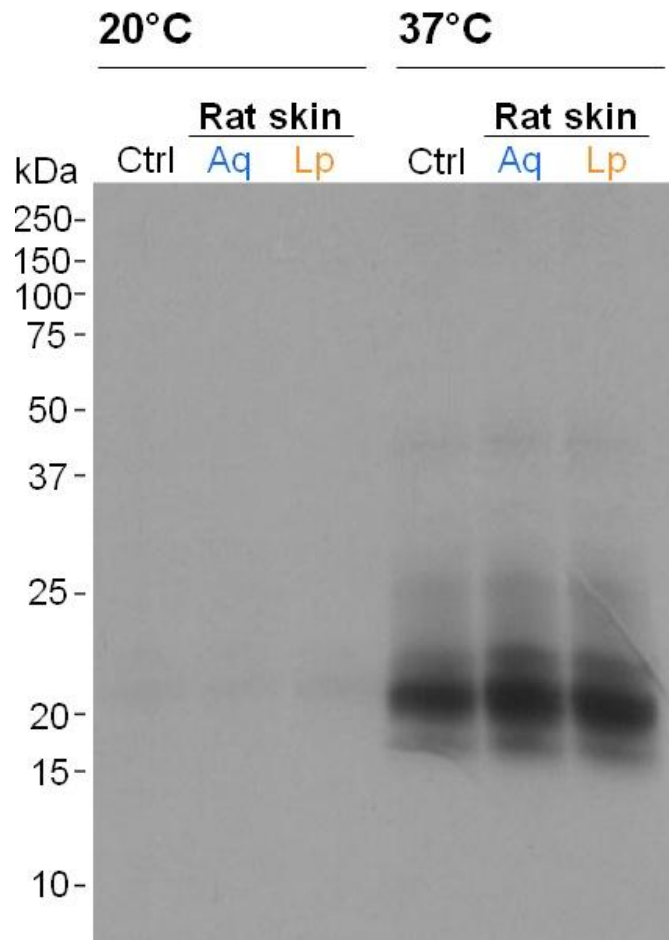


**Figure 4.8. Rat serum sensitisation shows no effect on the protein secretion profile of *N. brasiliensis* L3 (W strain) at 37°C.** L3 secreted proteins were metabolically labelled with  $^{35}\text{S}$ -Met, separated horizontally by isoelectric focusing and vertically by 10-20% gradient SDS-PAGE, then visualised by autoradiography. L3 were pre-sensitised with rat serum (+RS) where indicated. The amount of protein loaded was standardised to approximately 750,000 cpm per gel.

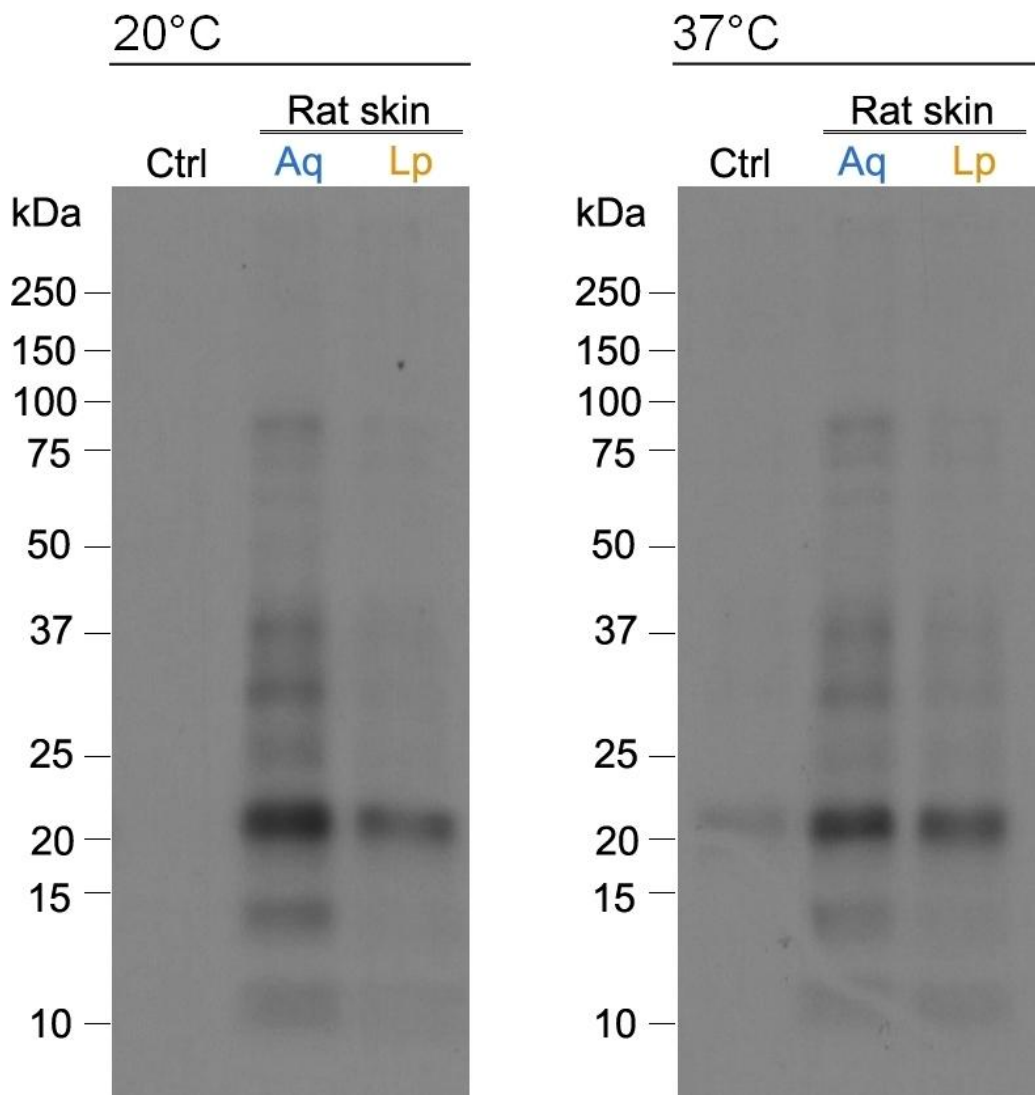


**Figure 4.9. Rat serum sensitisation shows no effect on the protein secretion profile of *N. brasiliensis* L3 (J strain) at 37°C.** L3 secreted proteins were metabolically labelled with  $^{35}\text{S}$ -Met, separated horizontally by isoelectric focusing and vertically by 10-20% gradient SDS-PAGE, then visualised by autoradiography. L3 were pre-sensitised with rat serum (+RS) where indicated. The amount of protein loaded was standardised to approximately 750,000 cpm per gel.

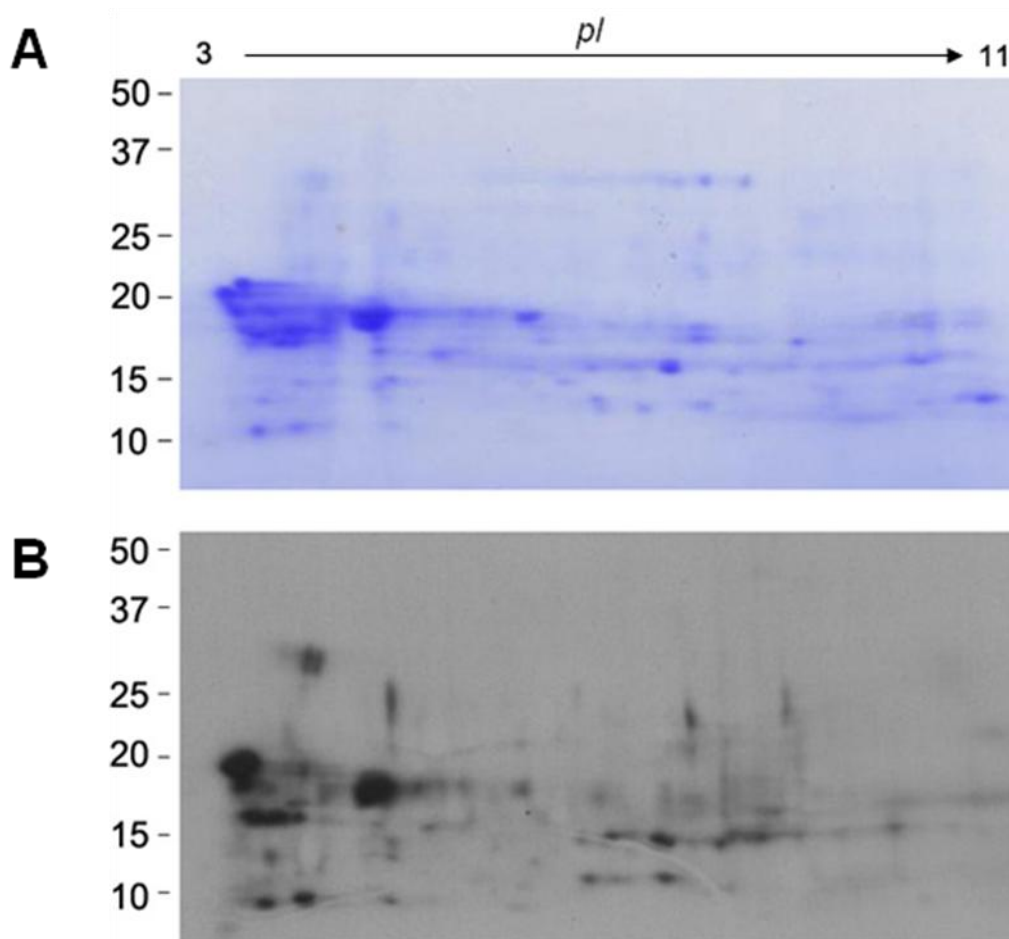




**Figure 4.10. Stimulation with rat skin fractions did not affect *de novo* production of proteins by L3.** L3 (J strain) were pre-sensitised with rat skin fractions for 24 hours, washed and cultured in fresh medium with  $^{35}\text{S}$ -Met for 2 days at 20°C or 37°C, so that newly synthesised proteins were metabolically labelled. The medium was collected, concentrated and resolved by 10-20% gradient PAGE.



**Figure 4.11. Release of pre-synthesised proteins by *N. brasiliensis* L3 is stimulated by rat skin fractions, independent of temperature.** *N. brasiliensis* L3 (J strain) were pre-labelled by cultivation at room temperature for 40 hours with  $^{35}\text{S}$ -methionine and stimulated with aqueous (Aq) or lipid (Lp) fractions extracted from 0.25 g of rat skin, at 20°C or 37°C for 30 minutes. The medium was collected, concentrated, resolved by 15% SDS-PAGE and visualised by autoradiography.



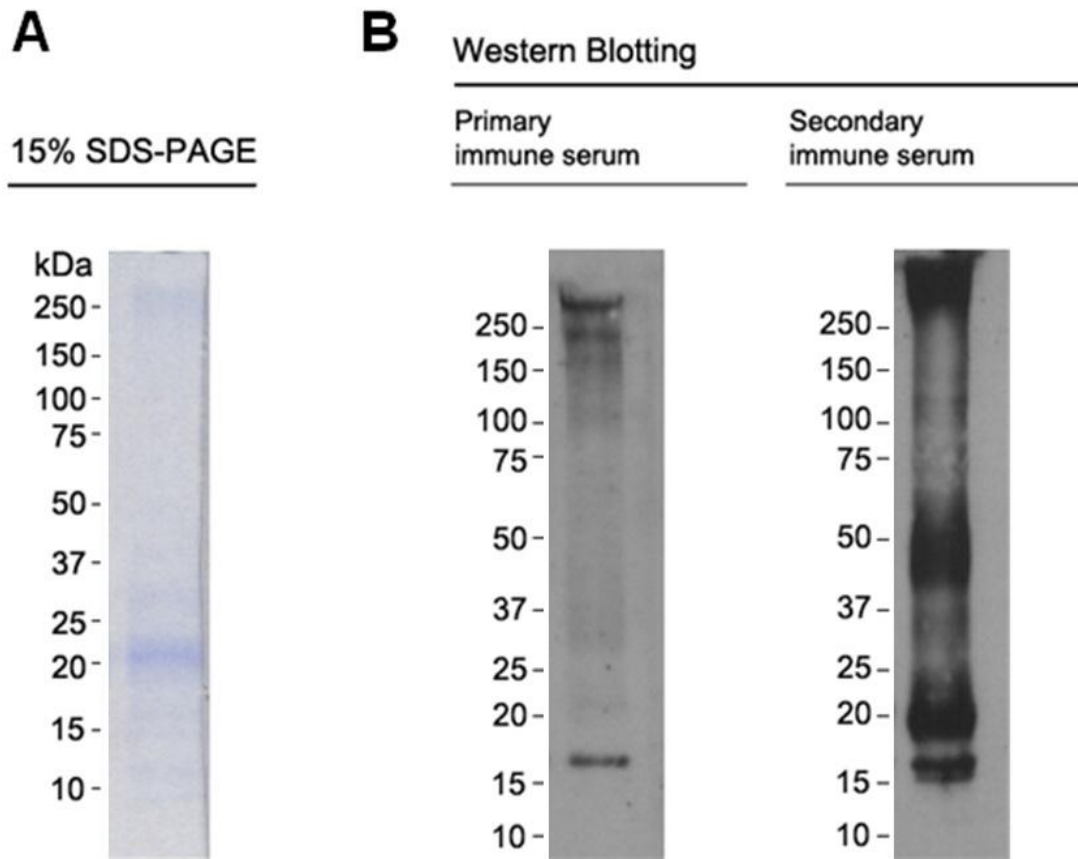
**Figure 4.12. Proteins released by stimulation with rat skin are the same as those released at 37°C.** A) Separation profile by 2-DE of L3 proteins secreted during cultivation at 37°C, visualised by Coomassie blue staining. B) Separation profile of pre-synthesised proteins stimulated by the aqueous fraction of rat skin, visualised by autoradiography.

#### 4.5. Immunogenicity of L3 proteins

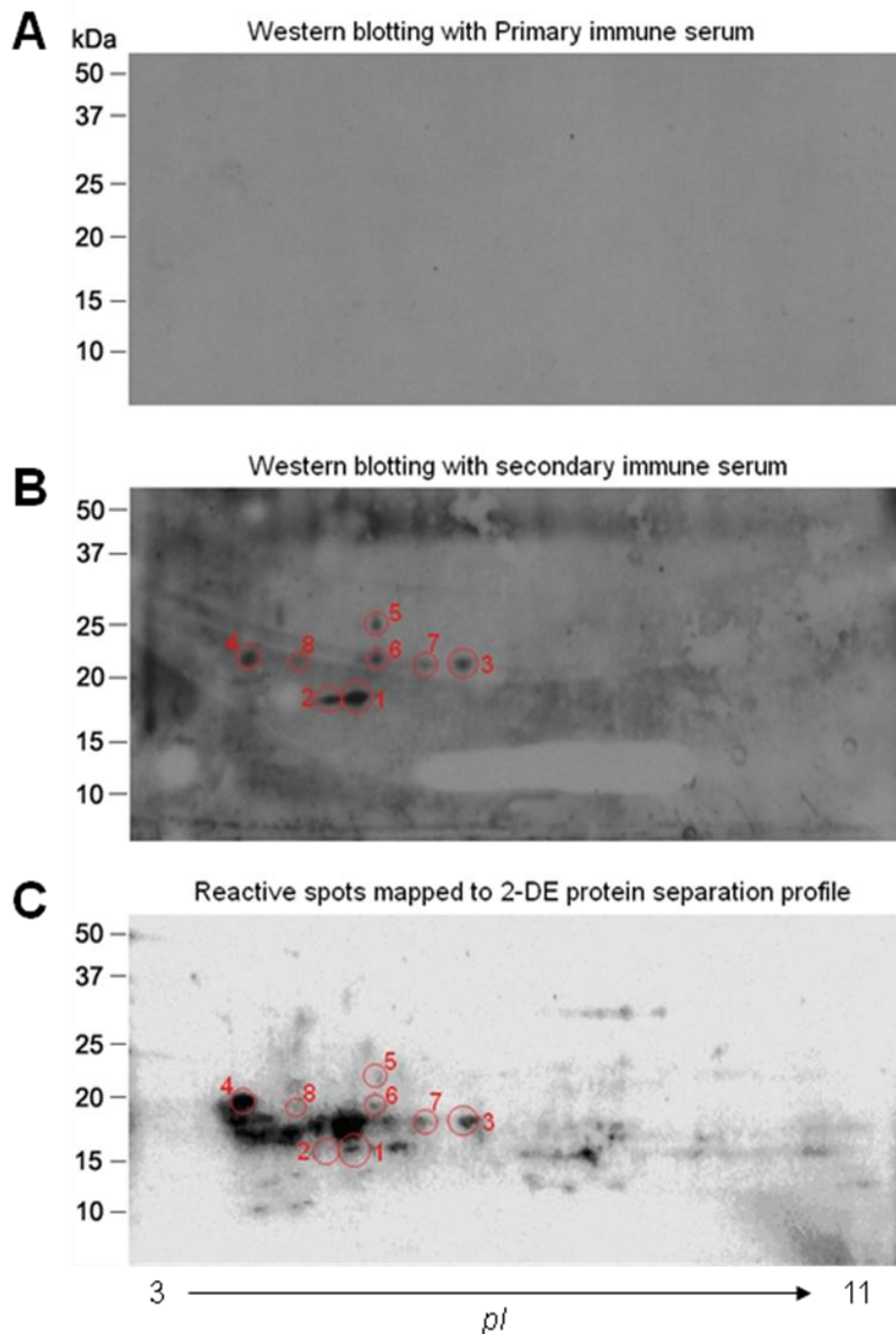
Proteins secreted by L3 through activation at 37°C (hereafter referred to as L3A secreted proteins) were resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes for Western blotting analysis. The proteins were reacted with sera collected from rats after a primary infection or a secondary infection (Fig. 4.13). Reactivity with the secondary immune serum was observed to be much stronger than that with the primary immune serum, indicating that immune recognition of L3A secreted proteins was increased by reinfection.

The L3A proteins secreted by the J strain was then resolved on 2-DE and analysed by Western blotting (Fig. 4.14). No reactivity was observed with the primary immune serum, but 8 distinct spots of reactivity were found with the secondary immune serum. The strength of reactivity was not correlated to the quantity of protein. For example, spot 1 and 2 on Fig. 4.14B, i.e. those with the highest reactivity, were mapped to very small protein spots on Fig. 4.14C. In contrast, spot 4 showed relatively weak reactivity, but represented a major protein. Their differential reactivity to immune serum suggests that these proteins would be interesting for future studies.

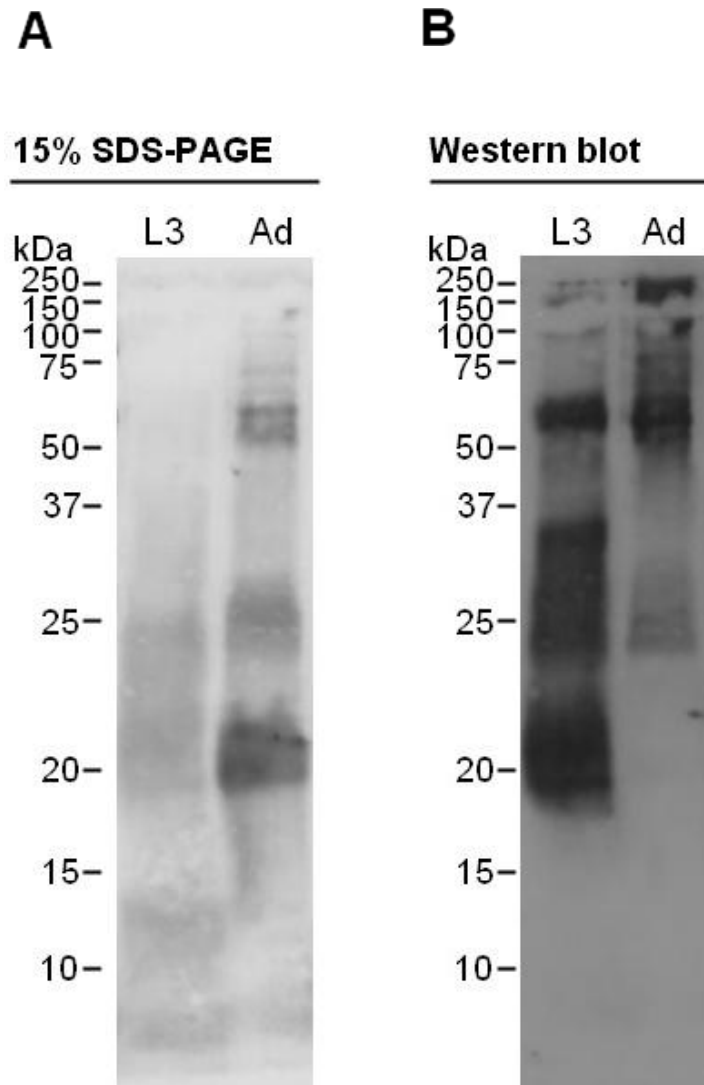
Proteins secreted by L3A show greater reactivity with hyperimmune serum than those secreted by adult worms, as shown by Western blotting analysis (Fig. 4.15). ELISA was carried out to determine the immunoglobulin isotypes directed against secreted proteins of L3 and adults during infection. Hyperimmune mouse serum was initially titrated to determine the optimal dilutions (Fig. 4.16). Sera from individual mice were then used at an appropriate concentration for each immunoglobulin isotype (Fig. 4.17). Background levels (assessed by reaction with naïve sera) were very low. Higher levels of IgG1 than IgG2a specific to both L3A and adult secreted proteins were found, indicating a Th2-biased profile. Comparable levels of IgA and IgE were also detected, which is a typical response to gastrointestinal nematode infection. Significantly higher levels of IgG1, IgG2a, IgG2b and IgE were observed to L3A secreted proteins in comparison to those from adult worms.



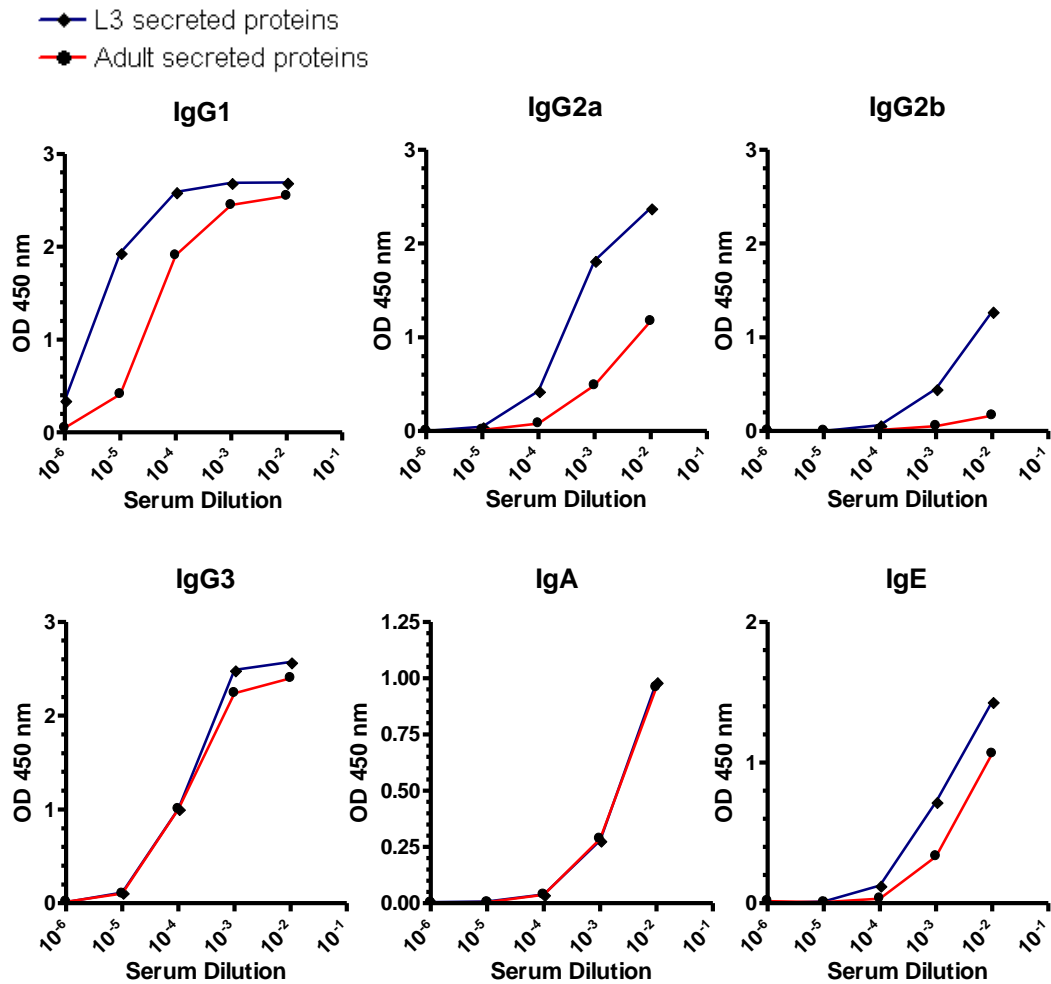
**Figure 4.13. Immunogenicity of L3A secreted proteins during infection.** L3A secreted proteins of the J strain was resolved A) by 15% SDS-PAGE and visualised by Coomassie blue staining, with 25  $\mu$ g of proteins loaded per lane. B) Western blotting analysis of L3A proteins, in which reactions were carried out with sera collected from rats after a primary or secondary infection at a dilution of 1:400. Anti-rat IgG-HRP secondary antibody was used (1:60,000).



**Figure 4.14.** *N. brasiliensis* L3A secreted proteins, separated by 2-DE, and reactivity with secondary immune serum. A) L3A secreted proteins resolved on 2-DE showed no reactivity to primary immune serum, but B) reacted specifically with secondary immune serum, resulting in 8 distinct reactive spots. Reaction was carried out with primary or secondary immune serum (1:400). Anti-rat IgG-HRP secondary antibody was used (1:60,000). C) The reactive spots (numbered 1-8, in descending order of reactivity) were mapped to the original 2-DE separation profile on the original nitrocellulose membrane visualised by DB71 staining.

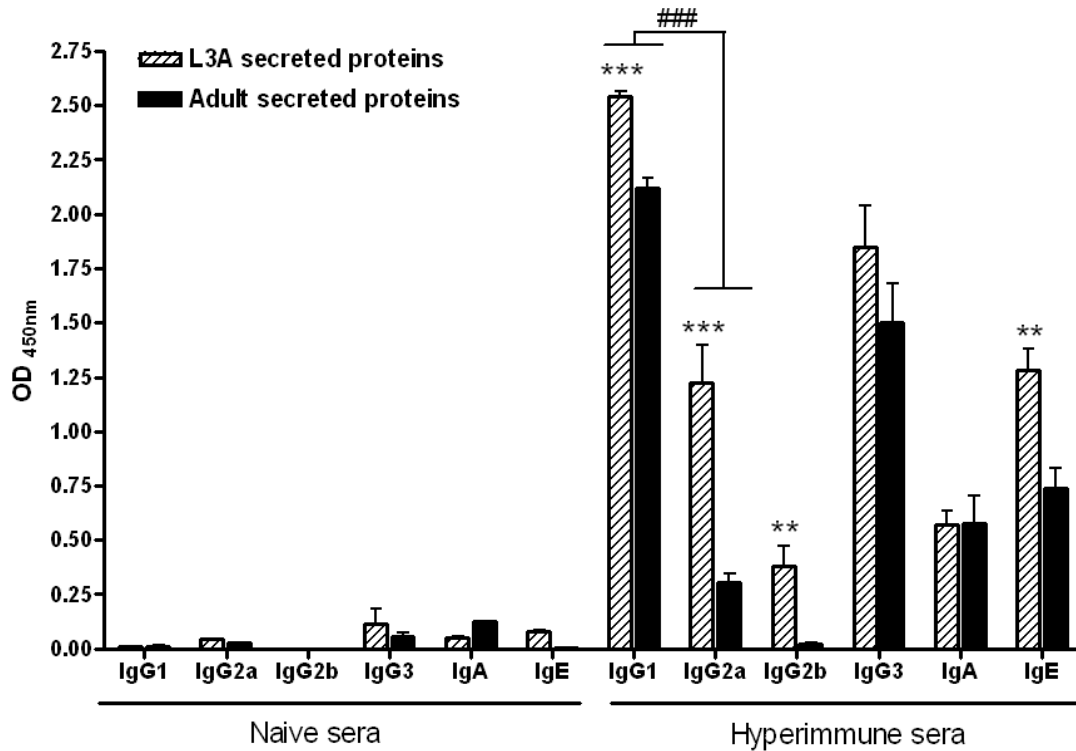


**Figure 4.15. Secreted proteins of L3A show greater reactivity with hyperimmune serum than those of adult worms.** A) Protein separation profile of L3A or adult (Ad) secreted proteins on 15% SDS-PAGE, in which 15  $\mu$ g of proteins was resolved per lane, visualised by DB71 staining. B) Western blot analysis of these proteins, reaction to hyperimmune serum (1:400) from mice infected 4 times with *N. brasiliensis*. Anti-mouse IgG-HRP secondary antibody was used at a dilution of 1:2000.



**Figure 4.16. Detection of L3 or adult-specific immunoglobulin isotypes in hyperimmune sera by ELISA.** Plates were coated with L3 or adult secreted proteins at 5  $\mu\text{g}/\text{ml}$  and reacted with hyperimmune serum pooled from 5 multiply infected mice, diluted to the indicated concentrations. HRP-conjugated secondary antibodies to immunoglobulin isotypes were used.





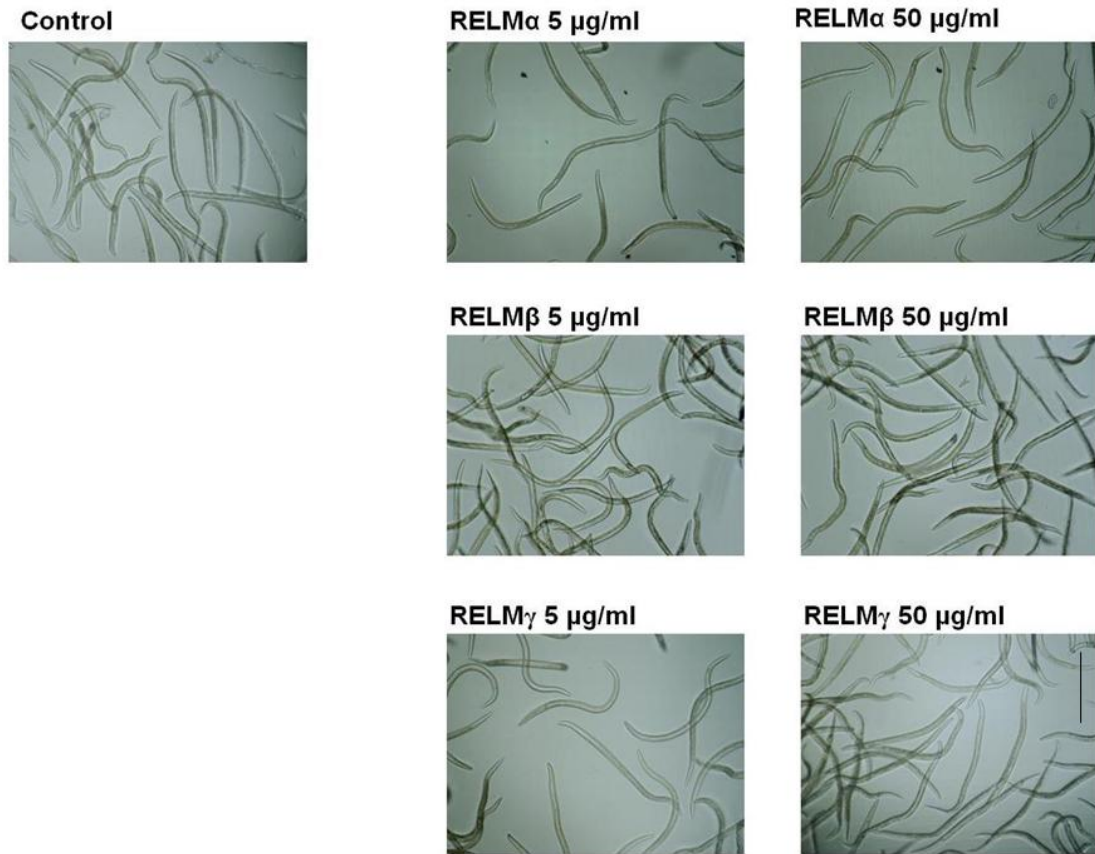
**Figure 4.17. L3 and adult-specific immunoglobulin isotypes in hyperimmune sera.** ELISA plates were coated with L3 or adult secreted proteins at 5  $\mu\text{g/ml}$  and reacted with sera collected from 5 individual naïve or multiply infected (hyperimmune) mice, each assayed in triplicate. Sera were diluted 1:100 for IgA and IgE detection and 1:1000 for detection of all IgG subclasses. HRP-conjugated secondary antibodies to immunoglobulin isotypes were used. Asterisks indicate a significant difference between reactivity to L3A and adult secreted proteins,  $***P < 0.001$ ,  $**P < 0.01$ . Hash signs indicate a significant difference between levels of antibody isotypes,  $### P < 0.001$ .

#### 4.6. The effect of RELMs on chemotaxis and feeding

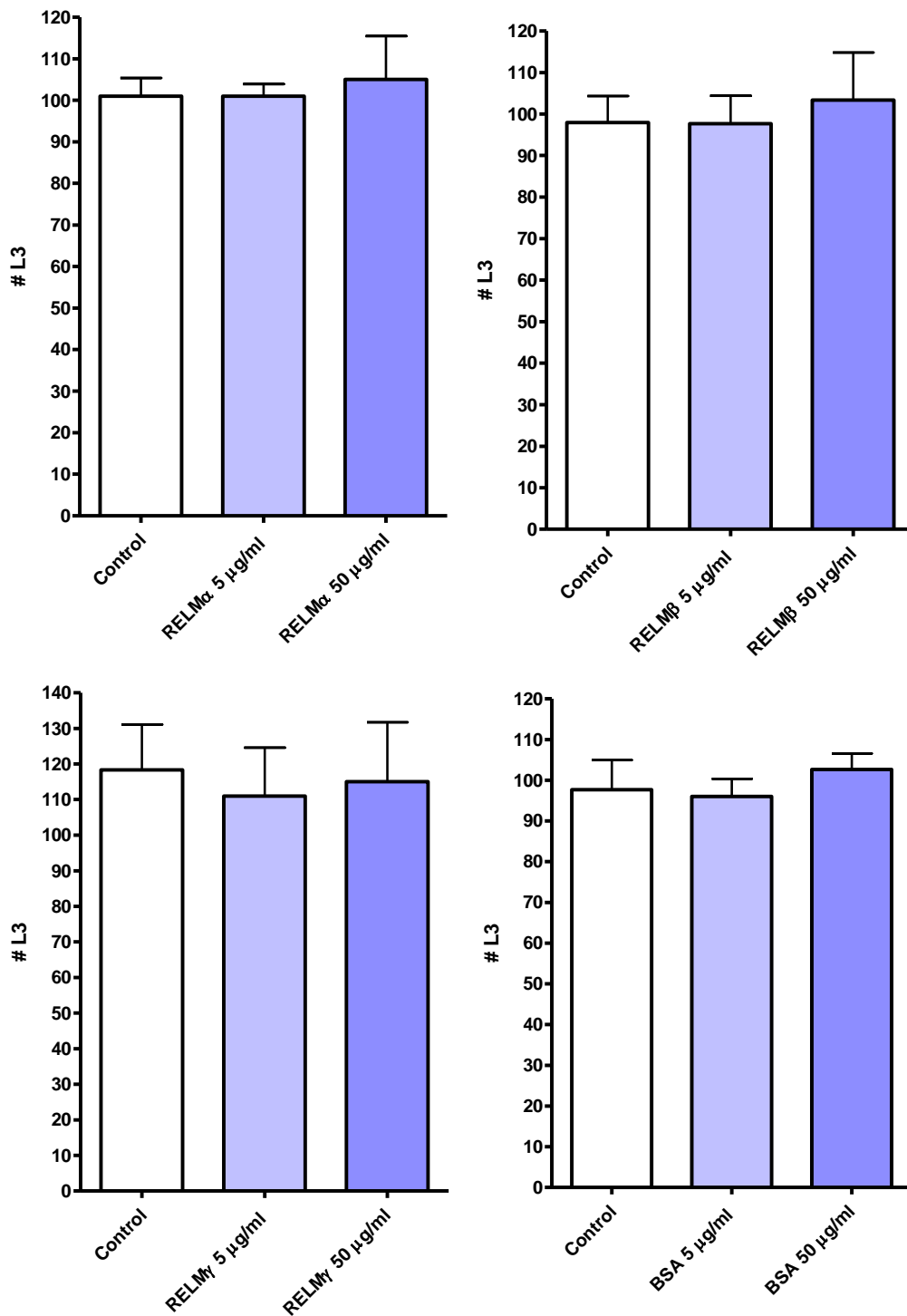
The REsistin-Like Molecules (RELMs) consist of a family of proteins secreted by mammalian cells including those of humans and rodents (Steppan *et al.*, 2001). In particular, RELM $\alpha$  and RELM $\beta$  have been shown to be relevant to nematode infection (Artis *et al.*, 2004; Pesce *et al.*, 2009). Furthermore, RELM $\beta$  has been shown to bind to chemosensory structures on *S. stercoralis* and inhibit larval chemotaxis in *S. stercoralis* (Artis *et al.*, 2004), as well as blocking feeding activities in the adult stage of *H. polygyrus* (Herbert *et al.*, 2009). Therefore, the focus of these experiments was to study the effect of RELMs on chemotaxis, activation and feeding of *N. brasiliensis*.

Incubation with RELM $\alpha$ ,  $\beta$  or  $\gamma$  did not seem to affect the general motility or appearance of L3 as observed by light microscopy (Fig. 4.18), nor did it affect larval migration towards an aqueous fraction of rat skin in chemotaxis assays (Fig. 4.19). The number of larvae which migrated towards rat skin was not affected when RELM $\alpha$ ,  $\beta$  or  $\gamma$  were used at 5  $\mu\text{g/ml}$  or 50  $\mu\text{g/ml}$ , the latter of which is the RELM $\beta$  concentration effective in inhibiting chemotaxis of *S. stercoralis* larvae (Artis *et al.*, 2004). Additionally, RELM $\beta$  also did not affect chemotaxis of L3 when a different substance, rat serum, was used as chemoattractant (Fig. 4.20).

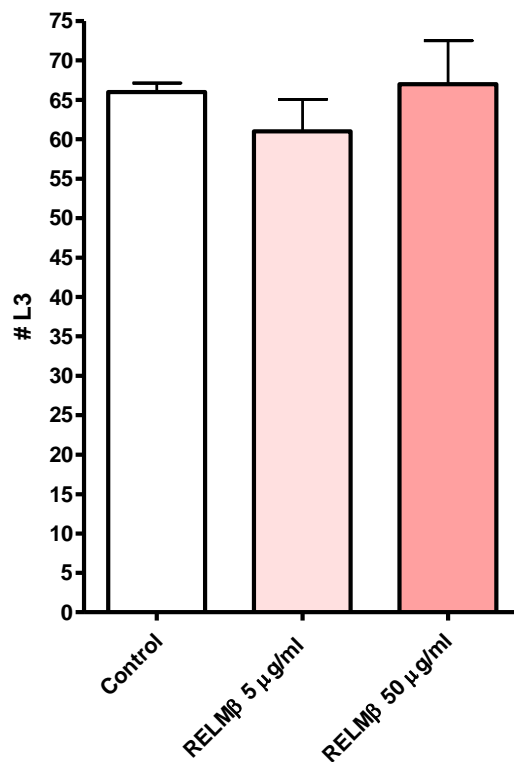
Assay with FITC-BSA showed that RELM $\beta$  had no effect on the activation of feeding in L3 (Fig. 4.21). Approximately 60-70% of larvae were activated to feed by 24 hours into cultivation at 37°C, regardless of the presence of RELM $\beta$  at 5  $\mu\text{g/ml}$  or 50  $\mu\text{g/ml}$ . To additionally assess the rate of feeding, L3A were fed with BSA radiolabelled with  $^{125}\text{I}$ , and the amount of BSA ingested was quantified by radioactive counts. RELM $\beta$  had no effect on the rate of feeding in L3A (Fig. 4.22). As the inhibition of feeding by RELM $\beta$  has been reported for the adult stage of *H. polygyrus*, I also sought to assess the rate of feeding in *N. brasiliensis* adults, although no significant difference was found between groups incubated with RELM $\beta$  and control (Fig. 4.23).



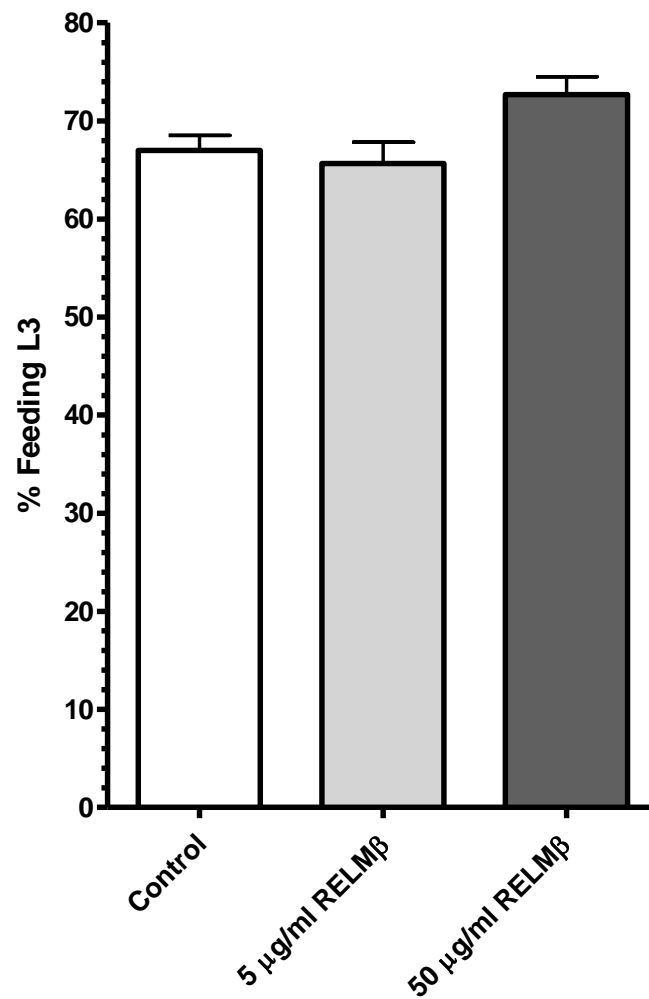
**Figure 4.18. RELM proteins do not have any overt effect on L3 motility.** L3 were observed under a light microscope after incubation with RELMs at room temperature for 3 hours. A negative control is also shown in which L3 were incubated with PBS only. L3 were photographed at x100 magnification. Scale bar (black): 400  $\mu$ m.



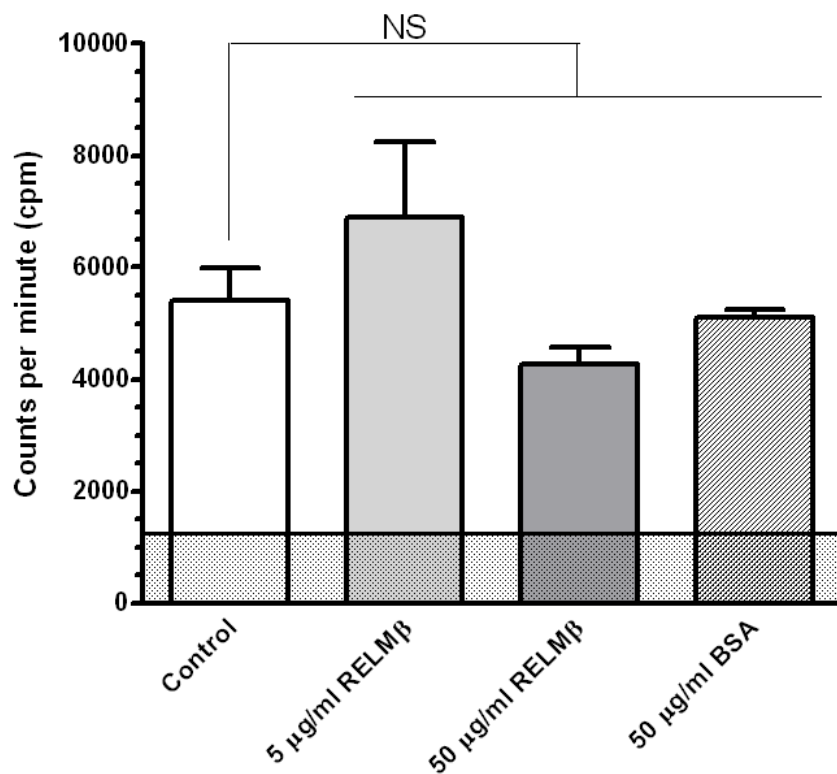
**Figure 4.19. RELMs do not affect chemotaxis of L3s to rat skin.** Prior to the assay, L3s were pre-incubated with RELM $\alpha/\beta/\gamma$  or BSA for 1 hour. Each bar show the number of L3s in the positive well (containing rat skin aqueous fraction) less those in the negative well (containing water), + 1 SEM, assayed in triplicate. Numbers were scored 120 minutes after addition of L3s. A negative control is also shown in which L3 were incubated with PBS only.



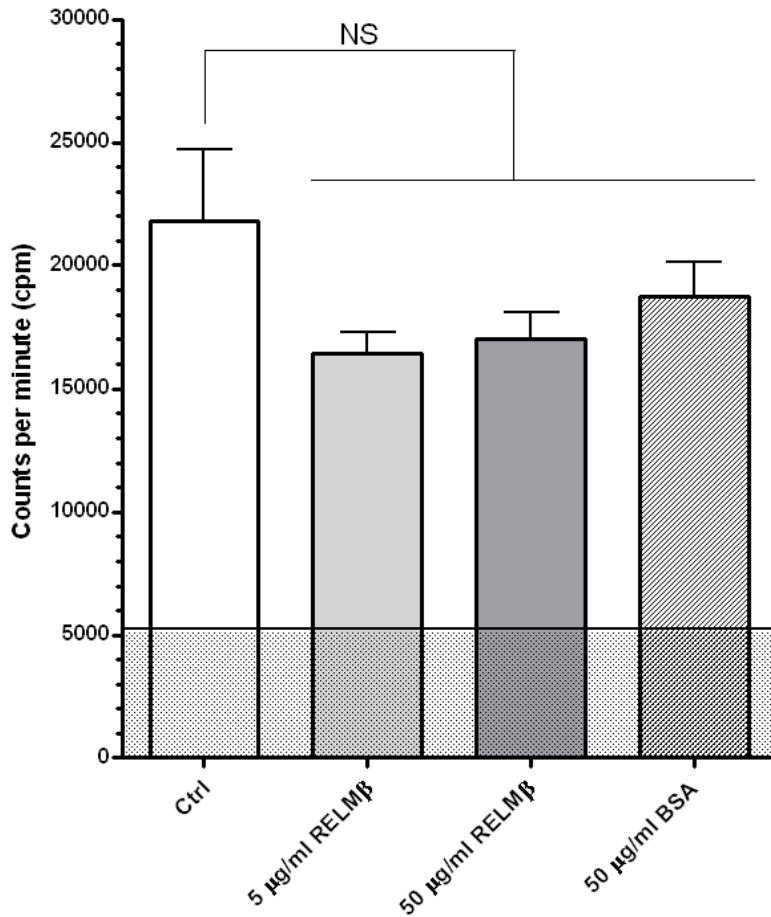
**Figure 4.20. RELM $\beta$  does not affect chemotaxis of L3s to rat serum.** Prior to the assay, L3s were pre-incubated with RELM $\beta$  or BSA for 1 hour. Each bar show the number of L3s in the positive well (containing rat serum) less those in the negative well (containing water), + 1 SEM, assayed in triplicate. Numbers were scored 120 minutes after addition of L3s. A negative control is also shown in which L3 were incubated with PBS only.



**Figure 4.21. RELMβ does not affect the activation of feeding in L3.** L3 were cultured for 24 hours, then incubated with RELMβ for 1 hour (where indicated) prior to feeding with FITC-BSA. Each bar shows the proportion of the larval population feeding at 24 hours post-incubation, + 1 SEM, in triplicate. A negative control is also shown in which L3 were incubated with PBS only.



**Figure 4.22. RELM $\beta$  does not affect the feeding rate of L3.** L3 were cultured for 24 hours, then incubated in RELM $\beta$  or BSA (where indicated) for 1 hour prior to feeding with  $^{125}\text{I}$ -BSA for another 2 hours. Each bar corresponds to the amount of  $^{125}\text{I}$ -BSA uptake of L3, + SEM, assayed in triplicate. A negative control is also shown in which L3 were incubated with PBS instead of the RELM molecules. Worms were heat-killed by incubation at 65°C for 10 minutes and assayed in parallel to the test conditions to assess background binding of  $^{125}\text{I}$ -BSA to the worm surface. This background value + 3 SEM was shaded at the bottom of the graph (1266 cpm).



**Figure 4.23. RELMβ does not affect the feeding rate of adult worms.** Adult worms were incubated in RELMβ or BSA (where indicated) for 1 hour prior to feeding with <sup>125</sup>I-BSA for another 2 hours. Each bar corresponds to the amount of <sup>125</sup>I-BSA uptake of L3, + SEM, assayed in triplicates. A negative control is also shown in which L3 were incubated with PBS instead of the RELM molecules. Worms were heat-killed by incubation at 65°C for 10 minutes and assayed in parallel to the test conditions to assess background binding of <sup>125</sup>I-BSA to the worm surface. This background value + 3 SEM was shaded at the bottom of the graph (5110 cpm).



## 4.7. Discussion

The factors for activation and protein secretion have been investigated in this chapter. In this section, aspects of these activities will be discussed with additional reference to nematode biology and the pathways which lead to these events.

### *Host finding and invasion of skin-penetrating nematodes*

The ability of the parasite to sense and respond appropriately to host signals is fundamental to successful host detection and invasion. Skin-penetrating nematodes have been found to be attracted to chemical factors derived from the skin. Like *N. brasiliensis*, infective larvae of *A. caninum* are attracted to aqueous extracts of host (dog) skin, but not the lipid fraction (Granzer and Haas, 1991). This finding is also true of *S. stercoralis* larvae, and through fractionation of the aqueous extracts, it was subsequently found that the major chemoattractant for *S. stercoralis* is urocanic acid, a chemical found in mammalian skin (Safer *et al.*, 2007). In these assays urocanic acid was used at 150 mM to demonstrate its chemotactic properties, which I replicated in addition to two other concentrations at 250 and 50 mM, although no significant concentration-dependent effects were found. This range encompasses the concentration of urocanic acid in normal human skin, which is approximately 60 mM (Safer *et al.*, 2007). Chemoattraction to this relatively generic compound is perhaps reflective of the wider host spectrum of *S. stercoralis*, which can infect a range of mammals including dogs, humans and other primates (Safer *et al.*, 2007). In contrast, *N. brasiliensis* has a much higher host specificity (normally infecting only rats), so it is likely that there exist other factors unique to rat skin that could serve as more specific entry cues for infective larvae. It would be an interesting project to elucidate the identity of such cues for *N. brasiliensis* in future work. Specificity of host recognition is particularly important for a parasite with a narrow host spectrum, since there may be fewer opportunities for infection, and entering a host which is not permissive to its development would be detrimental to its subsequent survival and propagation.

However, there also appear to be some common cues underlying host-finding and invasive behaviour in skin-penetrating nematodes. In addition to urocanic acid, *S. stercoralis* larvae are also sensitive to less specific host factors such as carbon dioxide and sodium chloride concentrations (Forbes *et al.*, 2003; Sciacca *et al.*, 2002). Carbon dioxide at physiological concentrations (3.3 - 4% in human breath) was found to induce active, non-directional crawling and waving in infective larvae, termed 'nictating' or 'questing', behaviour which seems to facilitate host detection and attachment of *A. caninum* and *S. stercoralis*, but not *H. contortus*, which normally infects the host occur via oral ingestion and so presumably does not require active host-seeking strategies (Sciacca *et al.*, 2002). Infective larvae of *Strongyloides ratti* and *S. stercoralis* migrate optimally towards a sodium chloride concentration of 30 – 70 mM (Forbes *et al.*, 2003; Tobata-Kudo *et al.*, 2000a), a range which suggests that they could be responsive to sweat (Patterson *et al.*, 2000). It is also very well-known that most parasitic nematodes of warm-blooded animals, such as *N. brasiliensis* and *Strongyloides ratti*, are strongly and positively thermotactic, and they show increased motility in response to warmth (Kassai, 1982; Tobata-Kudo *et al.*, 2000b). Infective larvae of the hookworms *A. caninum*, *N. americanus* and *A. duodenale* were shown to migrate sensitively to a zone of 37°C on a heat gradient, and they also exhibit turnback behaviour from lethal temperatures. It was additionally shown in these species that penetrative behaviour could be induced by heat alone (Granzer and Haas, 1991; Haas, 2003).

Interestingly, penetrative behaviour can also be induced in *A. caninum* L3 by aqueous skin extracts and serum (Granzer and Haas, 1991). This mirrors my results in that these substances also induced *N. brasiliensis* L3 to penetrate when a permissive medium (agar) was used. However, penetrative behaviour in *N. americanus* and *A. duodenale* were induced by fatty acids only (Haas *et al.*, 2005), suggesting that penetrative signals can differ even between closely related skin-invading nematodes. Although signals for host-finding and penetration may not be mutually exclusive, I believe that my findings may be more relevant to the latter, as chemoattraction may not be such an effective host finding strategy for a fast-moving host such as the rat. Indeed, analysis of the chemotactic properties of trematode cercariae showed that species which infect fast-moving host do not use chemotaxis for host finding, whereas those that infect slower moving targets such as snails do (Haas, 2003). Moreover, it is

also not clear how a chemical gradient of aqueous chemoattractant could be freely established from the intact skin of the host rat, leaving behind tracks for the infective larvae to actively follow. It is more likely that the larvae, abundantly positioned on the tips of grass stems, may be induced to become more active when a host is near (by less specific cues such as carbon dioxide levels or heat radiation) and would then attach passively to rat fur during a chance encounter, after which specific host cues may direct them towards entry sites on skin surface where they would be induced to penetrate towards the correct direction by chemo- and / or thermo-orientation.

### ***Activation of feeding and protein secretion in parasitic nematodes***

Activation is an intriguing process at the point which the parasite transits from its free-living form into a parasitic phase, and the study of it improves our understanding of the infective process. The *in vitro* fluorescent feeding assay developed by John Hawdon has been a useful tool in elucidating host factors which trigger activation, which was studied in most detail in *A. caninum*. The kinetics of activation appears to be similar between *A. caninum* and *N. brasiliensis*. Feeding activities were initiated *in vitro* in both species from 6 hours post-activation, and peaks at a feeding percentage of approximately 90%, although this can be achieved at an earlier time-point in *A. caninum* under serum and GSH-stimulated conditions (Hawdon and Schad, 1990, 1992).

The major differences were the activation signals for the two species. An elevation of temperature to 37°C was sufficient to induce feeding in *N. brasiliensis* L3 to 90%, whereas only 20% of *A. caninum* L3 could be induced to feed under these conditions (Hawdon and Schad, 1990). With addition of serum and GSH, the feeding percentage of *A. caninum* can be increased to 90% (Hawdon and Schad, 1992), whereas these compounds had no effect on the feeding of *N. brasiliensis* L3 at any time point (Huang *et al.*, 2010). Rat skin, either whole or fractionated, did not affect feeding, even though it was shown to be a potent chemoattractive signal for host-finding and / or penetration. A temperature cue at 37°C was also sufficient to stimulate synthesis and secretion of a complex mixture of proteins, again unaffected by serum or rat skin. These findings seem to suggest that a chemosensory input is not essential, but a

thermosensory input can suffice to induce activation in *N. brasiliensis* L3. This is somewhat surprising because nematode parasite activation is generally viewed as a complex event, sensitively dependent on physiochemical signals from the host. Nevertheless, it is notable that feeding and protein secretion represent a subset of the early events associated with activation, and the later, more complex events such as development to adults are likely to require more complex stimuli and cues.

Chemical exsheathment, which did not stimulate L3 to feed at 20°C and actually decreased feeding at 37°C, was found to have adverse effects on worm viability as shown by the MTT assay (Huang *et al.*, 2010), thus explaining the results. Nevertheless, it is not likely that initiation of feeding can be achieved solely by exsheathment of L3, as 1) Larvae which exsheathed naturally at room temperature did not show any feeding activities, and 2) the worms which remained viable after sodium hypochlorite treatment did not start feeding at an earlier time point than their unexsheathed counterparts. Moreover, nonfeeding L3 of *A. caninum* have been observed to exsheath in the absence of serum, suggesting that exsheathment and feeding are initiated by different stimuli (Hawdon and Schad, 1990). This is likely to be the case for *N. brasiliensis* L3 as well. In my experience, larvae freshly isolated from faecal cultures were usually a mixture of ensheathed and exsheathed worms, and there seems to be fewer ensheathed worms when older batches of faecal cultures were isolated, which suggests that *N. brasiliensis* L3 may be able to exsheath naturally at room temperature within the faeces, prior to activation.

L3 of *A. caninum* can also be activated to secrete proteins, but serum and GSH stimulation are required (Hawdon *et al.*, 1996). The fact that a temperature cue alone is sufficient to stimulate protein secretion in *N. brasiliensis* means that this is an ideal system for studying activation stimuli for protein secretion in larval parasitic nematodes. By cultivation at 37°C alone, clean and pure secreted proteins can be collected from supernatants, uncontaminated by foreign (e.g. serum) proteins. Through repeated batch culture, a comprehensive proteomics project could possibly yield fruitful data. A proteomics approach is likely to yield more realistic data for the study of secreted proteins than a transcriptomic approach (such as that carried out by Datu *et al.*, 2008), which, although informative, may not guarantee an infallible prediction of protein expression and secretion. The most abundantly secreted protein

found in *A. caninum* was named *Ancylostoma* secreted protein 1 (Hawdon *et al.*, 1996). Subsequently, it was found to belong to a large family of related proteins, with homologues in virtually all nematodes studied to date. We have found that these proteins are also secreted by *N. brasiliensis*, the study of which is the focus of Chapter 5 of this thesis.

The fact that *N. brasiliensis* L3 can release pre-synthesised proteins upon stimulation by rat skin suggests that chemosensory input is at least in part involved in some aspects of the early events of activation. Although protein pre-synthesis in the L3 stage has not been studied in parasitic nematodes, a precedent of this has been investigated in infective cercariae of the trematode *Schistosoma mansoni* (Newport *et al.*, 1988; Salter *et al.*, 2000). Host penetration by cercariae was found to be facilitated by a serine protease which was stored in their glands and released upon skin lipid stimulation. This means that important invasion factors need not necessarily be upregulated at activation, which additionally points to another weakness in the transcriptomic approach to the study of larval protein secretion. Indeed, transcripts of *Ac-MTP-1*, a metalloprotease which was shown to be one of the most abundantly secreted proteins upon activation and important for host invasion, was found in non-activated larvae as opposed to activated larvae (Datu *et al.*, 2008). It is very possible that nematodes may also be able to synthesise large amounts of proteins during their quiescent period – dauer larvae of *C. elegans* have been found to express thousands of genes despite their developmental and metabolic arrest (Fielenbach and Antebi, 2008). Theoretically, pre-synthesis of invasion factors would be a highly feasible strategy for nematode parasites of animal hosts, considering the narrow window of opportunity for invasion. The ability to immediately and abundantly release these proteins on host contact would be highly beneficial to the success of host colonisation.

Through Western blotting and ELISA analyses, it was found that L3A secreted proteins are highly immunogenic. Sera from animals which were multiply infected with *N. brasiliensis* showed higher immune recognition with L3A secreted proteins than with adult secreted proteins. This is most likely due to the fact that the majority of the parasites are killed at the lungs during reinfection (Harvie *et al.*, 2010), so that secreted factors of L3 are repeatedly exposed to the immune system while those of adults are not. This may have implications on the use of L3A proteins as vaccine

targets, as subsequent infections could augment natural immunity, creating a boosting effect on the memory response. Analysis of immunoreactivity of the proteins separated by 2-DE showed that the degree of recognition by immune serum was not correlated to the quantity of protein secreted, suggesting differential antigenic reactivity of the proteins with the immune system, as well as specificity of reaction. Such proteins may be interesting targets for further studies, as vaccination with these proteins may have the potential to generate a protective effect to infection. Some of the protective vaccine candidates identified from *H. contortus* secretory products, Hc25, Hc24 and GA1, reacted with hyperimmune sheep serum similarly when separated under 2-DE methods and analysed by Western blotting (Yatsuda *et al.*, 2003). Analysis of the antibody isotypes generated to *N. brasiliensis* secreted proteins during reinfection suggests that the response is Th2-biased, with greater amounts of antigen-specific IgG1 produced to IgG2a. Although antibody responses are generally unimportant in expulsion, the IgG1/IgG2a balance is often helpful in determining the type of T-helper response. IgG1 is upregulated by IL-4, a major Th2 cytokine in helminth infection, which downregulates IgG2a (Finkelman *et al.*, 1990). In contrast, IFN- $\gamma$  upregulates IgG2a (Snapper and Paul, 1987). Moreover, IL-4 also contributes heavily to class-switching to IgE, which showed an increase during infection with *N. brasiliensis* with antigen specificity to secreted proteins. It is known that helminth infection typically result in the production of IgE, although its protective properties have always been under debate. The production of antigen-specific IgE suggest that there may be some epitopes within the complement of secreted proteins which could have the potential to induce IgE-related hypersensitivity reactions, so that this should be kept in mind when considering vaccine candidates from the secreted origin.

### ***Neuronal control of chemotaxis, thermotaxis and developmental switching in nematodes***

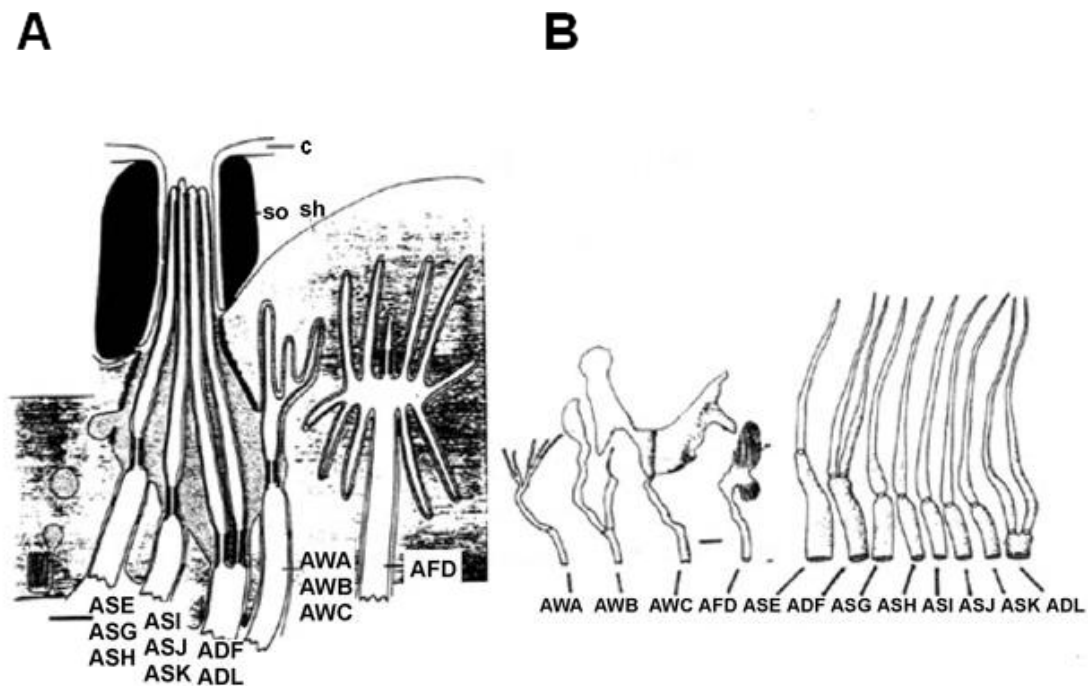
The ability of nematodes to sense and respond to host and environmental signals suggests that they possess functional sensory structures. Electron microscopy has revealed the neuronal structures of several nematodes, including *C. elegans* and *S. stercoralis* (Ashton and Schad, 1996). The structures, termed cuticular sensilla, are generally located in the head adjacent to the mouth. The tips of dendritic processes are

extended to pores in the nematode's cuticle, exposed to the external environment, which make them viable locations for sensory receptors involved in receiving signals from the environment exterior to ensheathed larvae. Of the cuticular sensilla, the amphidial neurones are the largest and most complex, and are the major sensory neurones of nematodes (Ashton *et al.*, 1999).

In *C. elegans*, there are twelve sets of bilaterally symmetric amphidial neurones. The structure of a generalised amphid is shown in Fig. 4.24. Through laser microbeam ablation studies, it was found that eleven of these are chemosensory (class ADF, ADL, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB and AWC), and one of them is thermosensory (class AFD) (Ashton *et al.*, 1999; Mori *et al.*, 2007). Most of these neurones end with a single or a double dendritic process, but two of them are structurally unique – the finger cell (AFD), with multiple digitiform dendritic structures, and the wing cell (AWA, AWB, AWC), with extended flattened processes (Fig. 4.24). The wing cell has also been shown to have olfactory properties, and are involved in the detection of volatile substances (Bargmann, 2006). In *S. stercoralis*, a neurone which consists of a large complex of lamellae (lamellar cell, ALD) was identified, and it appears to have physical characteristics of both the finger cell and wing cell (Ashton and Schad, 1996). Ablation of these lamellar cells resulted in impaired chemotaxis and thermotaxis, suggestive of sensory functions akin to the wing cell and the finger cell (Lopez *et al.*, 2000). Additionally, it was recently shown that directed chemotaxis of *S. stercoralis* can be induced when urocanic acid was presented as a volatile chemoattractant, suggesting olfactory input (Nyamu *et al.*, 2011). Structural homologues of the finger cell have been mapped in *H. contortus* and *A. caninum*, and in the latter they were again found to be the major thermoreceptors through laser ablation studies (Bhopale *et al.*, 2001). These cells are likely to play an important role in the thermoregulation of host-finding, invasion and developmental control. It is also likely that structural homologues of the chemosensory neurones in *C. elegans* would be functionally similar to those of parasitic nematodes, and indeed it was demonstrated that homologues of ASE and ASH mediate chemoattractive and chemorepulsive behaviour in *S. stercoralis* respectively (Forbes *et al.*, 2004).

Since the decision to enter or exit from developmental stages in nematodes is based on the detection of host signals or environmental conditions, it is not surprising that

amphidial neurones also play a major part in these processes. In *C. elegans*, ADF and ASI control development into dauer stage when conditions become unfavourable, and ASJ is primarily responsible for exit of the dauer stage and resumption of development (Bargmann and Horvitz, 1991). Parallels of this system were found in *S. stercoralis*, in which ablation of the ASF (equivalent to ADF in *C. elegans*) and ASI neurones prevented formation of its free-living, environmentally resistant stage (functionally comparable to the dauer stage in *C. elegans*) (Ashton *et al.*, 1998). Moreover, ablation of ASJ neurones resulted in disrupted activation of *S. stercoralis* infective L3 (Ashton *et al.*, 2007). These studies suggest that the neuronal control of development between free-living and parasitic nematode taxa may share some underlying similarities.



**Figure 4.24. Diagram of a generalised amphid.** A) Representation of amphidial neurones in *C. elegans*. Annotations: c, cuticle; so, socket; sh, sheath; AFD, finger cell; AWA, AWB, AWC, wing cells. B) Structural characteristics of the amphidial neurones. Nomenclature: The first letter A is for amphidial, second letter denotes a single (S) or double (D) dendritic process, or W for wing cell and F for finger cell. Diagram from Bargmann (2006).



### *Pathway to activation in nematodes*

Formation of an environmentally resistant, metabolically economical dormant state is a common strategy utilised by the free-living *C. elegans* and parasitic nematodes for prolonging survival in the face of adversity. Dauer formation in *C. elegans* is used for enduring unfavourable conditions such as extreme temperatures, high population density and food scarcity, whereas developmental arrest at the L3 stage is aimed at maximising its potential lifespan ('buying time') until a host is encountered. Both events occur at the dispersive (third) stage of their respective life cycles, to ensure maximisation of reproductive output. However, for *C. elegans* entry into the dauer state is a conscious decision (Hotez *et al.*, 1993; Riddle and Albert, 1997), as evident by mutants which are dauer defective in certain signalling components, which are unable to enter the dauer stage even when conditions are unfavourable (Fielenbach and Antebi, 2008). In contrast, the formation of developmentally arrested L3 appears to be a default option for parasitic nematodes (Hotez *et al.*, 1993), which is intuitive because the parasite would rarely have the good fortune to encounter a host immediately after maturation to its infective stage. Within the context of developmental switching, *C. elegans* and parasitic nematodes share in common a trigger mechanism in the exit of the dauer and L3 stage respectively, an event which is of particular interest to parasitologists because it would give insight of the events during invasion, which may be relevant to intervention strategies for diseases.

Dauer recovery in *C. elegans* is regulated by converging transforming growth factor-beta (TGF- $\beta$ ) and insulin-like signalling pathways (Fig. 4.25) (Beall and Pearce, 2002; Fielenbach and Antebi, 2008), activated when environmental conditions are favourable, resulting in the inactivation of negative regulators to genes which promote growth and metabolism, such as those involved in the synthesis of steroid hormones on which reproductive growth is dependent (Antebi *et al.*, 2000; Motola *et al.*, 2006). The TGF- $\beta$  signalling pathway is initiated by increased food supply and low pheromone levels, as perceived by the ASI neurone. This activates the production of a small molecule called dauer-formation (DAF)-7, a TGF- $\beta$ -like ligand to the DAF-1/DAF-4 receptor kinase complex (Kimura *et al.*, 1997; Ren *et al.*, 1996). Binding results in phosphorylation of the downstream effector Smads, DAF-8 and DAF-14, which translocate to the nucleus and antagonise DAF-3, a negative regulator of genes

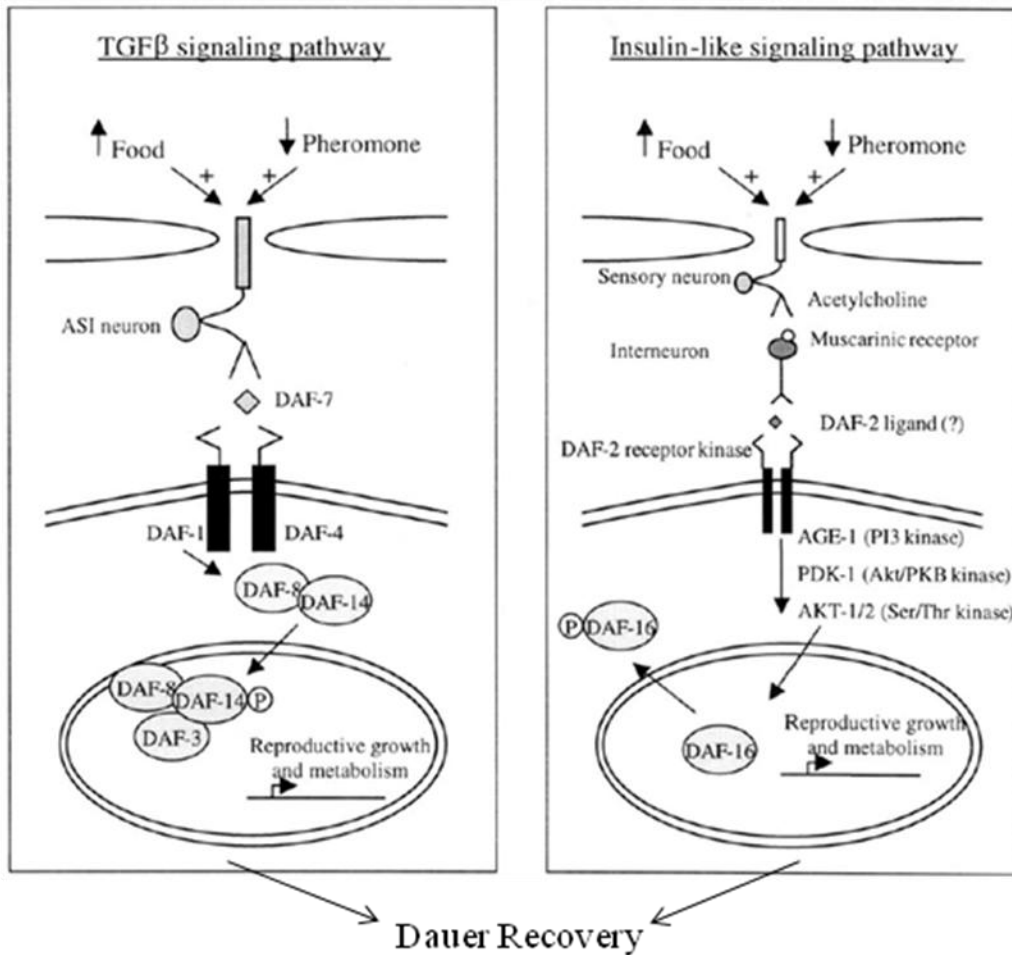
that promote growth and metabolism. Similarly, signalling through the insulin-like pathway is also dependent on the perception of favourable environmental signals via a sensory neurone that releases acetylcholine (ACh), which then signals through a muscarinic receptor to produce a ligand for the DAF-2 receptor kinase complex (Braeckman *et al.*, 2001). Binding initiates a phosphoinositide 3 (PI3)-kinase signalling cascade, which results in phosphorylation of the negative regulator DAF-16, thus inactivating it and allowing for transcription of genes that promote growth and metabolism.

Fueled by this knowledge, studies were then carried out to investigate the pathway to activation in *A. caninum* L3. Thanks to the ubiquity of cholinergic signalling, activation can be studied through the use of agonists and antagonists to this pathway. Recovery from the L3 stage was found to be induced by muscarinic ACh receptor agonists and inhibited by atropine, a muscarinic antagonist (Tissenbaum *et al.*, 2000). Inhibition of the PI3K signalling cascade also prevents activation in L3 of *A. caninum* and *A. ceylanicum* (Brand and Hawdon, 2004). These data demonstrate the importance of cholinergic input and suggests that an insulin-like signalling pathway akin to that responsible for dauer recovery is involved in activation. Furthermore, this pathway was found to be preceded by cyclic GMP (cGMP) signalling in both *A. caninum* and *C. elegans*, collectively suggesting a common pathway for dauer recovery and *A. caninum* activation (Birnby *et al.*, 2000; Hawdon and Datu, 2003). Recently, the signalling pathway leading to activation in *N. brasiliensis* has been studied in our laboratory (Huang *et al.*). Unlike *A. caninum* and *C. elegans*, cGMP and cholinergic signalling are not required for activation of *N. brasiliensis* L3, but functional PI3K signalling is required. These data suggest that there are substantial differences between the early signalling events which lead to the activation of *A. caninum* and *N. brasiliensis*, but the pathways may converge downstream of PI3K signalling.

Although *daf-7* homologues have been identified in several species of parasitic nematodes, there has been no substantial evidence that the TGF- $\beta$  signalling pathway is involved in activation and L3 recovery (Viney *et al.*, 2005). Temporally, *daf-7* is expressed in pre-dauer larvae (L1 and L2) of *C. elegans* under favourable environmental conditions, but not in the dauer larvae themselves until stimulated to

resume development (Ren *et al.*, 1996). However, *daf-7* homologues were found to be predominantly expressed in the resting, infective L3 stage of most parasitic nematodes including *S. ratti*, *S. stercoralis*, *Parastrongyloides trichosuri*, *H. contortus*, *N. brasiliensis* and *A. caninum* (Brand *et al.*, 2005; Crook *et al.*, 2005; Freitas and Arasu, 2005; Massey *et al.*, 2005; McSorley *et al.*, 2010), which is counterintuitive to the view that expression of *daf-7* induces resumption of growth and development. The mRNA levels of *daf-7* were found to be decreased in *S. ratti* and *P. trichosuri* upon host-induced activation (Crook *et al.*, 2005), which lead some researchers to the hypothesis that the role of DAF-7 in parasitic nematodes is to maintain the state of developmental arrest, the opposite of its function in *C. elegans*. In addition, the expression of *daf-7* is unaltered in *A. caninum* L3 before and after serum-stimulated activation (Brand *et al.*, 2005), a finding which further dissociates its involvement with the L3 recovery process.

The study of activation in parasitic nematodes is technically challenging because it is an event which normally occurs within the host. The *in vitro* activation assay has so far allowed study of control of some early events, namely resumption of feeding, exsheathment, protein synthesis and secretion. However, thus far it has not been possible to induce development of L3 to adult worms *in vitro*, a process which is probably sensitively dependent on successive exposure to complex host factors. Comprehensive knowledge of the signalling pathways leading to dauer recovery comes from the ability to generate mutants in *C. elegans*, in addition to being a free-living species, which allows for all stages of its development to be studied. An obvious solution would be the utilisation of RNAi to produce and study the knock-down phenotypes, however this technique has yet to be fully developed in parasitic nematodes. Until then, *C. elegans* will continue to be an invaluable tool for the modelling of the biology of parasitic nematodes, although qualified by potential differences.



**Figure 4.25. Schematic overview of the converging TGF- $\beta$  and insulin-like signalling pathway in *C. elegans*.** Activation of these pathways result in transcription of genes involved in growth and development, and subsequently dauer recovery (Beall and Pearce, 2002).

## **RELMs**

Experimentation on the effect of RELMs on chemotaxis of *N. brasiliensis* L3 stems from the observation that RELM $\beta$  binds to the bacillary and cuticular pores of *T. muris* and *S. stercoralis* larvae (possible sites of chemosensory reception), and the demonstration that RELM $\beta$  impairs chemotaxis of *S. stercoralis* larvae *in vitro* (Artis *et al.*, 2004). This effect was not replicated in my experiments with *N. brasiliensis* L3. The effect of other members of the RELM family was additionally tested in the chemotaxis assay, as they are highly related by sequence homology, but they also did not affect larval chemotaxis. Furthermore, RELM $\beta$  also did not have an effect on activation or the rate of feeding of *N. brasiliensis* L3, suggesting that larval activities are generally unaffected by RELM $\beta$ .

The RELMs belong to a family of resistin-like proteins expressed in mammalian cells. RELM $\alpha$  and RELM $\beta$ , in particular, have been shown to be involved in immunity to parasitic nematodes. RELM $\beta$  is expressed exclusively by intestinal goblet cells (Steppan *et al.*, 2001), and its expression was found to be highly upregulated after infection with *T. muris*, *T. spiralis* and *N. brasiliensis* at the time of expulsion (Artis *et al.*, 2004). In another independent study, RELM $\beta$  was also found to be upregulated at day 7 post-infection with *N. brasiliensis*, along with other genes which are thought to contribute to expulsion (Yamauchi *et al.*, 2006). Mice knocked-out for the RELM $\beta$  gene experience delayed expulsion of *N. brasiliensis*, and the intestinal worm numbers of *H. polygyrus* were also decreased (Herbert *et al.*, 2009). These lines of evidence suggest that RELM $\beta$  functions at the intestinal surface, and is likely to target adult worms rather than larvae. It was further demonstrated that RELM $\beta$  inhibits feeding in the adult worms of *H. polygyrus*, subsequently decreasing their ATP content and survival in the gut (Herbert *et al.*, 2009). However, RELM $\beta$  also did not affect adult feeding in *N. brasiliensis* in my experiments, perhaps due to a difference in worm biology or experimental protocol.

### ***Chapter conclusion***

Although the characteristics of larval activation were similar between the nematode species such as *A. caninum* and *N. brasiliensis*, which were both initiated to feed, exsheath and secrete proteins, the activation stimuli and signalling pathway which leads to this event can show substantial differences. The definition and identification of proteins secreted by L3 during activation is expected to yield interesting insights into the mechanisms of the infectious process and host-parasite interactions during the early events of infection, and therefore deserves further attention.

# **Chapter 5**

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## **VAL proteins in *N. brasiliensis***

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## 5.1. Introduction

Venom allergen homologue / *Ancylostoma* secreted protein-like (VAL) proteins are a major group of nematode secreted proteins, found in all parasitic nematodes studied to date. Expression patterns of VALs are often stage-specific, and are generally upregulated at infection or at later parasitic stages. VAL proteins have been shown to exhibit immunogenicity across various systems, and are considered as important vaccine candidates. In *N. brasiliensis* we have discovered eight variants of the VAL proteins, and their expression patterns, immunogenicity and suitability for vaccination will be the focus of this chapter.

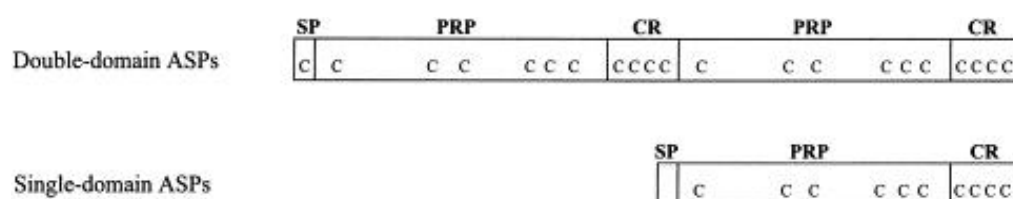
VALs belong to a family of cysteine-rich proteins collectively known as the SCP/TAPS family. Members of this family share a common primary structure, characterised by a signal peptide followed by a signature SCP-extracellular domain. Based on sequence homology, these proteins have been identified across a number of eukaryotic taxa from a phylogenetically diverse variety of animals, plants, fungi, helminths and insects (Cantacessi *et al.*, 2009). Various groups of these proteins are thought to have biological roles in host-pathogen interactions, of which the best characterised include the plant pathogenesis-related proteins (PRPs) which are often upregulated in response to pathogen-induced injury or other stress (Van Loon *et al.*, 2006), and the vespid venom allergen proteins, major components of the venom of biting insects which cause allergic reactions (King and Spangfort, 2000). Other prominent members include the glioma pathogenesis-related protein (GliPR) (Murphy *et al.*, 1995), the sperm coating glycoproteins (SCP) (Jalkanen *et al.*, 2005), mammalian testis-specific protein (Kasahara *et al.*, 1989), along with other members of the cysteine-rich secretory protein (CRISP) superfamily of mammals (Gibbs *et al.*, 2008) The widespread distribution of the SCP/TAPS proteins suggests that they serve diverse but critical functions.

In parasitic nematodes, these proteins were first described in the dog hookworm *Ancylostoma caninum*. Like *N. brasiliensis*, unstimulated *A. caninum* L3 secrete virtually no proteins, but become activated when exposed to host-specific factors (Hawdon and Schad, 1990, 1992), as previously described in Chapter 4. The most abundantly secreted proteins upon activation were resolved at molecular weights of



24 and 45 kDa, and were named *Ancylostoma* secreted proteins (ASP)-1 and ASP-2 respectively (Hawdon *et al.*, 1996; Hawdon *et al.*, 1999).

The authors went on to discover four more members of ASPs in *A. caninum*, ASP-3 to ASP-6 (Zhan *et al.*, 2003). Comparison of their amino acid sequences revealed a common theme in the domain structure of the ASPs (illustrated in Fig. 5.1). All members possess an N-terminal signal peptide, followed by either a single or a double PRP domain. A cysteine-rich (CR) region follows each domain, which forms the joining hinge of the molecule in the case of double domain ASPs. At least 10 conserved cysteines were found in single domain ASPs, and over 20 in double domain ASPs. The molecular weight of a single domain lies generally between 20-25 kDa, making a double domain protein 40-50 kDa (Zhan *et al.*, 2003). In a later study, it was found that the VAL proteins constitute the major upregulated transcripts in activated *A. caninum* L3 larvae (Datu *et al.*, 2008).



**Figure 5.1. Primary structure of double and single domain ASPs.** SP, signal peptide; PRP, pathogenesis-related protein domain; CR, cysteine-rich region; C, conserved cysteine residues. Figure from Zhan *et al.* (2003).

Bioinformatic and phylogenetic analyses have since revealed a great number of VAL homologues in a broad range of nematodes parasitic in animals and plants, as well as in the free-living species *C. elegans* (Zhan *et al.*, 2003). Multiple variants of VALs commonly exist in a given species; for example at least three variants have been found in *N. americanus*, two in *A. duodenale*, fifteen in *O. ostertagi* (Visser *et al.*, 2008), thirteen in *S. mansoni* (Chalmers *et al.*, 2008), three in *Onchocerca volvulus* (Tawe *et al.*, 2000) and at least seventeen in *C. elegans* (Zhan *et al.*, 2003). Most of

them possess either a double domain or a C-type single domain (a single domain protein with higher homology to the C-terminus of the prototype double domain VAL) (Visser *et al.*, 2008).

The discovery of these proteins results from a convergent effort by researchers working on different helminth species. Database searching had often revealed well-characterised proteins from other systems as VAL homologues – such was the case for the Hc24 and Hc40 proteins of *H. contortus* – whose immunogenic properties have been discovered and used as vaccine candidates years before the description of the *A. caninum* ASPs (Rehman and Jasmer, 1998; Schallig *et al.*, 1994; Sharp *et al.*, 1996). This, however, has resulted in some inconsistencies regarding the nomenclature of these proteins, for example some of them were named ASP-like (AL) based on their homology to the ASPs, or activation-associated secreted protein (ASP), while others were named venom allergen-like (VAL), venom allergen homologues (VAH) or venom allergen proteins (VAP) based on the homology with the major allergens in the venom of the yellow jacket wasp (Henriksen *et al.*, 2001), with many others given unrelated names. They will hereon be collectively called VALs, standing for Venom allergen homologue / Ancylostoma secreted protein-Like proteins.

The functions of VAL proteins in parasitic helminths are still largely unclear. Based on their activation-associated properties seen in *A. caninum* L3 under host factor-stimulated conditions, it is frequently speculated that they are important molecules in the transition to parasitism from an environmental existence and the infection process. In support of this, it was found that antibodies to *Na*-ASP2 from *N. americanus* were able to inhibit larval migration *in vitro*, although no protease activity has been found for this protein (Bethony *et al.*, 2005; Goud *et al.*, 2004). Although it is not known whether *B. malayi* infective larvae secrete VALs, *Bm*-VAL-1 was detected in L3 extracts (Murray *et al.*, 2001) and has been shown to be secreted by microfilariae (Rees-Roberts, 2007). In *O. volvulus*, the filarial nematode which causes river blindness, *Ov*ASP-1 is one of the most abundant larval transcripts (Tawe *et al.*, 2000). Three members of *Ov*ASPs were subsequently found, and their expression was shown to be developmentally distinct; *Ov*ASP-3 is L3-specific, *Ov*ASP-2 is expressed at all stages, whereas *Ov*ASP-1 was produced by L2, L3, moulting L3 and adult females. *Ov*ASP-1 and 2 have been suggested to have a role in pathogenesis and survival, by

promoting angiogenesis to increase blood supply and support the nodules in which the parasites reside, but this would however be a function more relevant to microfilaria (which can migrate to the eye and causes ocular onchocerciasis) and adults (which reside within nodules in subcutaneous tissue) (Higazi *et al.*, 2003; Tawe *et al.*, 2000). Infective L3 on the other hand, migrate through skin until they mature as adults, so increased vascularisation is not particularly beneficial to this stage.

Data supporting a proinflammatory role for VALs have been presented for *Na*-ASP-2, which shows structural and charge similarities to the CC-chemokines as determined by x-ray crystallography (Asojo *et al.*, 2005). *Na*-ASP-2 was found to induce recruitment of exclusively neutrophils and monocytes using an *in vivo* air-pouch model and an *in vitro* chemotactic (Boyden chamber) assay (Bower *et al.*, 2008). The authors suggest that *Na*-ASP-2 may act as a chemokine mimic, and they speculate that this could possibly favour larval invasion by manifesting higher tissue permeability and oedema. However, it has been criticised that the level of chemotaxis shown in the data is low, with a chemotactic index (number of cells which migrated in test conditions divided by the number of cells migrated in medium alone) of only 1.5 – 2. Moreover, the idea that migrating larvae should actively recruit neutrophils in aid of tissue digestion is somewhat counterintuitive, and has not been widely accepted by the nematode research community.

Alternatively, VALs have been suggested to act as antagonistic ligands to complement receptor 3 (CR3, Mac-1, Mol, CD11b/CD18), an integrin expressed on the surface of neutrophils, monocytes, NK cells and macrophages (Arnaout, 1990), thereby blocking leukocyte adhesion to endothelial cells and pointing towards an anti-inflammatory role instead. One of the best functionally characterised members of the nematode VALs is the neutrophil inhibitory factor (NIF) secreted by *A. caninum*. *Ac*NIF is a 41 kDa protein shown to bind to granulocytes and monocytes, specifically to the CD11b domain of CR3, subsequently blocking adhesion to vascular endothelial cells as well as hydrogen peroxide release by activated human neutrophils (Moyle *et al.*, 1994; Rieu *et al.*, 1995). A later study showed that *Ac*NIF can block leukocyte adhesion through the inhibition of CD11a as well as CD11b (Lo *et al.*, 1999). NIF has additionally been shown to inhibit neutrophil recruitment in a model of acute lung injury in guinea pigs (Barnard *et al.*, 1995) and during LPS-induced cell infiltration

into the lungs of mice (Zhou *et al.*, 1998), subsequently reducing inflammatory insult. A 55 kDa NIF has also been identified in *H. contortus* adult secretory products, which cross-reacts with an antibody raised against AcNIF and showed similar inhibitory functions (Anbu and Joshi, 2008). Notably, vaccination with NIF conferred protection to hamsters from *A. ceylanicum* in terms of impairing parasite fecundity (Ali *et al.*, 2001). Also showing high sequence homology to NIF and the ASPs is the hookworm platelet inhibitor (HPI) protein, which inhibits the aggregation and adhesion of platelets to collagen and fibrinogen (Chadderdon and Cappello, 1999; Del Valle *et al.*, 2003). HPI is secreted by the blood-feeding adult stage hookworms in the gut, and they are therefore potentially useful targets for vaccine strategies aimed at inhibiting hookworm feeding.

Although a wealth of data has been generated on VAL proteins across multiple systems, to date no definitive function can be established for the nematode VAL proteins. Admittedly, with the consideration that at least seventeen VAL homologues exist in free-living *C. elegans* (Zhan *et al.*, 2003) comes the argument that VAL proteins may function in general nematode housekeeping rather than in parasitism. It could also be the case that the VAL proteins serve diverse functions even within the phylum nematoda through divergent evolution.

Despite their elusive functions, the VAL proteins of many parasitic nematodes show promising immunogenic properties. Natural antibodies produced in hosts after multiple infections often show reactivity with VALs, which means that an adaptive immune response can be mounted by these proteins. This was shown in *H. contortus* for Hc24 and its related proteins, which displayed a range of reactivity to hyperimmune sera, and protective effects were also conferred by vaccination with these molecules (Schallig *et al.*, 1994; Yatsuda *et al.*, 2003). *B. malayi* BmVAL-1 is recognised by the sera of >95% of human filariasis patients (Murray *et al.*, 2001). A VAL orthologue secreted by *O. ostertagi* is also recognised by antibodies collected from abomasal mucus and draining lymph nodes of hyperimmune calves (De Maere *et al.*, 2002). In addition, studies on protective fractions against *O. ostertagi* and *A. caninum* have also indicated VAL homologues as the immunodominant antigens (Fujiwara *et al.*, 2006; Geldhof *et al.*, 2003).

These promising results attracted the interest of developing VAL proteins as vaccine candidates. The human hookworm vaccine initiative was found in 2000, forming the first effort at a clinical trial towards a hookworm vaccine. It aims to formulate an injectable bivalent vaccine composing of two hookworm antigens - one from the larval stage and one from the adult stage, as targeting the parasite at both stages is likely to provide a higher level of protection. *Na*-ASP-2 of *N. americanus* was chosen as the larval antigen according to the following rationale: 1) ASP-2 is abundantly secreted during hookworm activation upon exposure to host factors, and protective parasite fractions are enriched in ASP-2 (Geldhof *et al.*, 2003; Hawdon *et al.*, 1999); 2) Vaccination with ASP-2 resulted in significantly reduced hookworm burden upon parasite challenge in hamsters and dogs (Bethony *et al.*, 2005; Goud *et al.*, 2004); 3) Antibodies raised in rats and dogs against ASP-2 were able to inhibit larval penetration of host skin *in vitro* (Bethony *et al.*, 2005; Goud *et al.*, 2005); 4) a cross-sectional serological and parasitological survey of human subjects from hookworm endemic areas in Brazil and China revealed a reciprocal association between high anti-ASP-2 immunoglobulin levels and the risk of heavy hookworm infection (Bethony *et al.*, 2005).

Phase I clinical trials were conducted with *Na*-ASP-2 expressed in *Pichia pastoris*, formulated with Alhydrogel as adjuvant, in healthy hookworm naïve human volunteers, inducing high humoral and cellular responses (Bethony *et al.*, 2008b). Although the study concluded with the description that the vaccine was ‘well tolerated’ and ‘without any significant vaccine-related adverse effects’, it was observed that 3 out of the 36 participants were excused from the study after the first and third boost due to ‘mild adverse reactions’. Vaccination with *Na*-ASP-2 also induced measurable amounts of IgE, which was consistently higher than controls, although not statistically significant. The authors went on to test the vaccine in previously infected individuals in Brazil, in which adverse hypersensitivity reactions immediately became evident. Within 2 hours of vaccination, 3 out of 7 volunteers injected with the *Na*-ASP-2 vaccine developed generalised urticaria (Bethony *et al.*, 2008a). It was found that the affected individuals all had higher *Na*-ASP-2-specific IgE prior to the vaccination, and they all responded to treatment with antihistamine. This shows that the *Na*-ASP-2 antigen can induce immediate-type hypersensitivity in

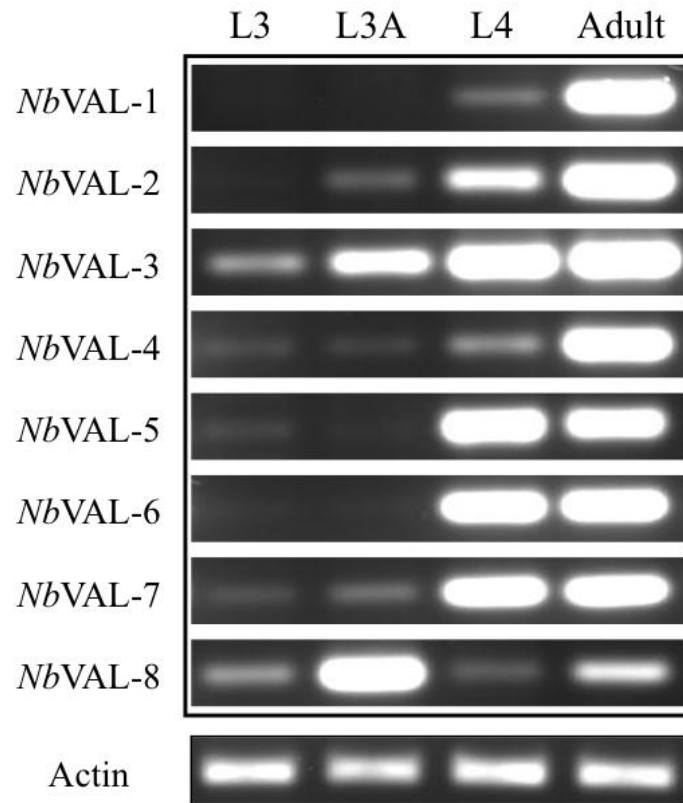
previously infected individuals, which include the main target population of the vaccine. Human trials for the *Na*-ASP-2 vaccine are currently on hold (Periago, 2010).

Previous work in our lab (Huang, 2010) has identified eight variants of VAL proteins in *N. brasiliensis* by searching EST databases using *Ac*ASP-1 as the query sequence, and their full length sequences were obtained by rapid amplification of 5'/3' cDNA ends (5' and 3'-RACE). The eight variants were named *Nb*VAL1 to *Nb*VAL8, and they show stage-specific transcription. All were transcribed by the adult stage as shown by reverse transcription-polymerase chain reaction analysis (RT-PCR) (Fig. 5.2). *Nb*VAL8 and *Nb*VAL3 were abundantly transcribed by L3, whereas *Nb*VAL7 and *Nb*VAL2 were also transcribed but at a lower level. Sequence analysis showed that they follow the same domain structure with the prototype VALs, with an N-terminal signal peptide, followed by a single or a double PRP domain joined by a cysteine-rich hinge (Fig. 5.3). The presence of a signal peptide in all eight variants suggests that they are all secreted proteins, but their secretion would need to be confirmed by further experimentation. Four of the *Nb*VALs have single domains (*Nb*VAL1, 4, 5 and 6), while the other four possess double domains (*Nb*VAL2, 3, 7 and 8). Cysteine residues were highly conserved (shaded yellow in Fig. 5.3), and they also align well with VAL homologues in different nematode species (Huang, 2010). Work in our lab has focused on *Nb*VAL3 to *Nb*VAL8, and work on *Nb*VAL1 and *Nb*VAL2 is being carried out by our collaborators, the Maizels lab.

The full length cDNA of single domain *Nb*VALs and the N-terminal domain of the double domain *Nb*VALs, with the signal peptide sequences removed, were amplified and cloned into the pET29b expression vector, then verified to be in frame with a poly-histidine tag at the C-terminal end. The plasmids were successfully transformed into *E. coli* BL21(DE3) cells, and expression of *Nb*VAL3-8 could induced at a high level with IPTG. However, the expressed products were mostly insoluble. The insolubility of the *Nb*VALs is most probably due to the high number of disulphide bonds, making correct folding in the cytosol difficult. This problem has been common with most researchers working on VAL proteins (Hotez *et al.*, 2003).

Therefore, one of the aims of this project was to establish a protocol to produce the *Nb*VALs in a soluble form, which has a higher chance of being correctly folded and

could be used in a much wider range of experimentation. The next aims were to analyse the immunogenicity of the *Nb*VAL proteins, the outcome of vaccination with recombinant proteins, and to study whether they, like their counterparts in *N. amercianus*, might have the tendency to induce Type I hypersensitivity.



**Figure 5.2. Stage-specific transcription of *NbVALs* analysed by RT-PCR.** Total RNA was isolated from L3, activated L3 (L3A), L4 and adult stages of *N. brasiliensis*, and was amplified by RT-PCR with primers specific for each *NbVAL* variant. Primers for actin used as a positive control to determine relative transcript levels (Huang, 2010).



# A

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NbVAL-2 1 .MAVLRVCCGSIQSSKNSSSLQSLAVRLLPAAQDTLWGFSNNDTNRNTEFLTTLNAAE
NbVAL-3 1 .....MRWLVVLLVYTPLVAAIQSKFNCPSDLGQQDDTREEFLKRHNIR
NbVAL-7 1 .....MAPPGRVFLIVVIVENAVAVGHEYHCNKDLIMNDDLRFNMLNYHNDIR
NbVAL-8 1 MPRLWFVYCRNFSLFLSLAILAVAVLSRTNGTQNFNCQNSANSEMTEDELRAYLNFHNNQR

NbVAL-2 60 NNTALGYIPIANGRGYLPTTSNMNLEMYDCFLEAKAYQQVQGC.....AKAETDDSV..Y
NbVAL-3 46 QNTALGMLAVNYFSYFLGPAKNMYQLEWSCDLERIAKATINQC.....QFKNVSTK...
NbVAL-7 48 TNLAWG...AQDWPTGQGPKNMYRVGWDCKLEEEAAKSLQCC.....SEAEYTAAPNNE
NbVAL-8 61 RRLARGNSPARSGK..FNKAKNMYKLEWSCSIEELAKRAVANCNADLT.ENRSYQNLAGY

NbVAL-2 113 FVLDATQSGKC DILAEIASVVN..KSFYDSGLQIDTNPB...RFWKAEQADF AKIANADAG
NbVAL-3 97 .YVYNTNKFWHYGFENVRAIEEGVYQWVEPIHMNDFGPK...FECHDKTREFCNMAYPTNT
NbVAL-7 100 AVLDVISKPRNKWPATNFALHRAIINSWYKPSIGFDQGST...L.ADEKIGAFGNMINYKAV
NbVAL-8 119 TFWSSDGNVPRSHIKMKELIKKTELESWENQAKEQGWKDPNN.KFTNSGIYAFANMANSKTT

NbVAL-2 169 LVGCFQKYC...GNDLHISCFEDKGLAINSTDRTKAT...VIYAPGKTALCDATEGSTADS
NbVAL-3 154 RLGCAYERC...GSELLISCAAYEEGKVPFKPILELAPHFCVCKHYPNACDEDYLCRATS
NbVAL-7 157 RLGC SYKVC...GDKVHVRCIYNDGANKVGD TLYEDG...WVC...YSLS DCTTYENSRCSG
NbVAL-8 179 ELGCAHNICKG.NTRVQILCLYNRVGTFTGASLYRTG...KPCQKNSDCTVFPQSTCDG

Cysteine-rich hinge

NbVAL-2 224 TGGNTGLCIRGAPKPAITTSSTTTTTTTTTTTR.S.CPNITGSSNDYMRNYFLNQHNQYR
NbVAL-3 212 INTTLEPLGPATIRTTITTRATTTTAPKLDVPKAS.CPTLNNKMTDEIRQLFDTHNKWR
NbVAL-7 210 ..GLCEAPDNLVLRTTTTT...TTTTANPSAGGDGNSQC.ANNPEMTDEARQMLTDKHNQIR
NbVAL-8 234 ....HGLCIKKRENPD DKTNIM.....CPRSSTMIDKVRQKFLNLHNHYR

NbVAL-2 283 SSIAQGNVYKVTGTNVR..QASRMSRMTYSCTLEQNAYDWARQCINKKSPGSDRYENIYA
NbVAL-3 272 SILVARGKAEDKLSPGGYA.PKASRMKPLWYDCKLEE SSKQVAEKCAN.KHSYGDYGENLEA
NbVAL-7 266 AQTAKGLSVDPKSATGFA.PKGSAMKMKYDCETEASAQAYTNLCKGLQHSWGQYGNITWM
NbVAL-8 276 SILVATGRTYDKLLGKN.T.PKAARKMQLKYSCALEATAWRYSHNVDLIPIPGKKNAGENL

NbVAL-2 342 HTDTSLARREVARLAMERWWRQITNNGLPANHIFVDKKGTT.NMLSKTIYDEGRRVGC SVV
NbVAL-3 331 ASFSGYDQDEVAKMAVDQWAGELKKEFGVDNLIYPTPGLT..GHYTQVWVQNTTRVGCYIN
NbVAL-7 326 IFAKNYNRKDVADWPPHSWFDELKEYGVGEKNIENASMMNV.GHYTQVWVGDTRFGCGFK
NbVAL-8 335 YKNLFLFDKLGPRCRLKTGLKRSKLLVWVWSGQFLHEIFRF.RPLHADVWQAPT TWMCNPR

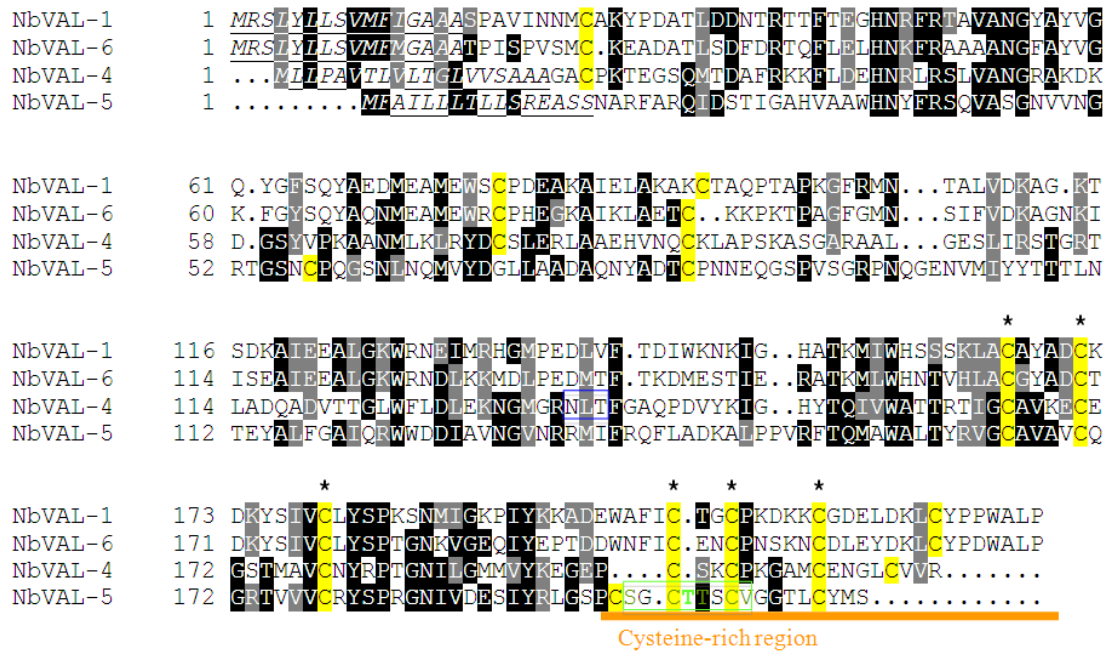
NbVAL-2 401 K.....CSTFTIAVCRYDPAGPAFNTQYYLLGAA CNRCD.GRPCSAGLCG.....
NbVAL-3 390 DECFFPDSAGWKTQVVCQYKPTGNFMRWEVYSKGEPCKN.DSDCKCKNCSCLAE EGLCYDP.
NbVAL-7 386 S.G...AGSGYTALICQYAPPGNWLDSTIYKVGEPSCACFPVGTKCEDGALCA.....
NbVAL-8 395 .....LPRYALVVCPOK.GGKLDPEVYTAGSPSCQCGNSELCNPNEGLCSAVVTFPG.

NbVAL-2 .....
NbVAL-3 449 NSMAYI..
NbVAL-7 .....
NbVAL-8 447 EGIKPGMQ

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See next page for figure legend

## B



**Figure 5.3. Amino acid sequence alignment of A) double and B) single domain NbVALs.** Identical/similar amino acids are shaded in black/grey. Cysteine residues are highlighted in yellow. Conserved cysteines are marked with an asterisk above the indicated positions. Predicted signal peptide sequences are in underline-italics. The cysteine-rich (CR) / hinge region is indicated with an orange bar below the sequences. Figure is adapted from Huang (2010). Additionally, putative N-linked glycosylation motifs are boxed in blue, and asparagines residues predicted to be N-glycosylated are additionally coloured in red (analysed on NetNGlyc). Putative O-linked glycosylation patterns are highlighted and boxed in green, and predicted residues for O-glycosylation are bold in lime green (analysed on OGPET).

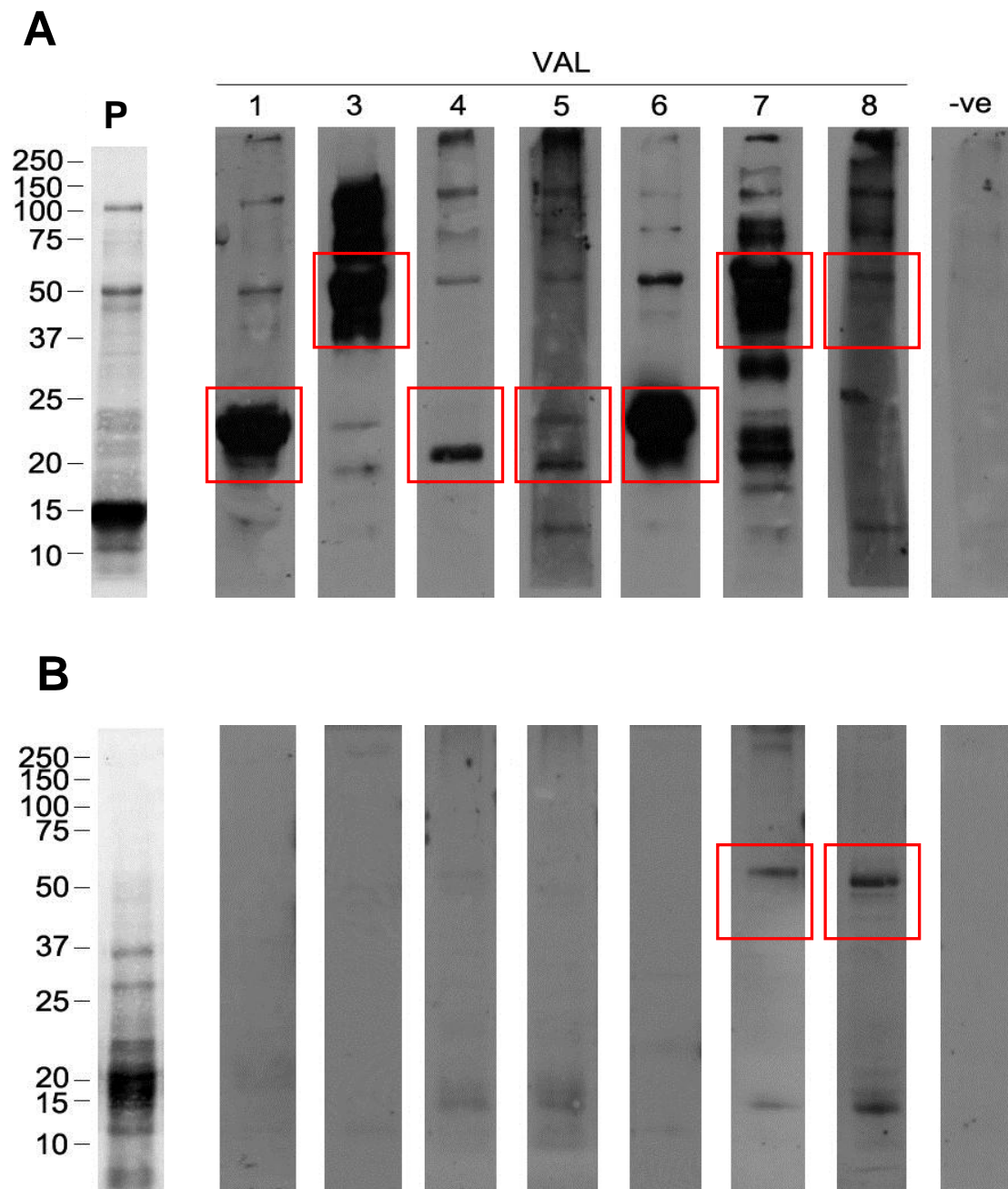
## 5.2. Analysis of recombinant *NbVAL*s expressed in *E. coli* BL21(DE3) cells

As mentioned, recombinant proteins of *NbVAL*3-8 have been successfully cloned into pET29b plasmids, expressed in *E. coli* BL21(DE3) cells, and purified from the insoluble fractions. Each *NbVAL* protein was used to immunise mice for the generation of antisera. The antiserum to *NbVAL*1 was provided by the Maizels lab, and antiserum to *NbVAL*2 has not been produced.

The antisera for *NbVAL*1 and *NbVAL*3-8 were individually used to identify native proteins in the secreted proteins of *N. brasiliensis* adult and L3 by Western blotting (Fig. 5.4). A pre-bleed mouse serum was used as a negative control, which did not bind to L3 or adult secreted proteins. The antisera showed good general reactivity with adult secreted proteins, recognising each single or double domain *NbVAL* at its predicted size (Fig. 5.4A, squared in red). The reactivity of anti-*NbVAL*5 and anti-*NbVAL*8 with adult secreted proteins were slightly questionable, as the reactions were weaker than others. Other bands were also observed on the blots at the incorrect sizes, due probably to non-specific binding and perhaps cross-reactivity to the other *NbVAL* variants.

The antisera to *NbVAL*7 and *NbVAL*8 showed reactivity to L3 secreted proteins at about 50 kDa, their predicted size (Fig. 5.4B). However, *NbVAL*3 was not detected in L3 secreted proteins, despite its high transcription levels shown by the RT-PCR results (Fig. 5.2). This suggests that there could be some post-transcriptional processing involved which inhibited the expression of *NbVAL*3, or that the protein is expressed but not secreted. The latter was found to be untrue, as the antiserum did not show specific reactivity with the somatic extracts of L3 (Huang, 2010).

Taken together with the RT-PCR results, it is determined that *NbVAL*1 and *NbVAL*3-8 are all transcribed and highly likely to be secreted by the adult worms, and *NbVAL*7 and *NbVAL*8 are additionally secreted at the L3 stage. This experiment also showed that the antisera generated by vaccination with VAL proteins produced in BL21(DE3) cells, although insoluble and probably incorrectly folded, are capable of recognising the native protein. However, due to high similarity between the *NbVAL* variants, the possibility of cross-reactivity between the VAL variants cannot be excluded.



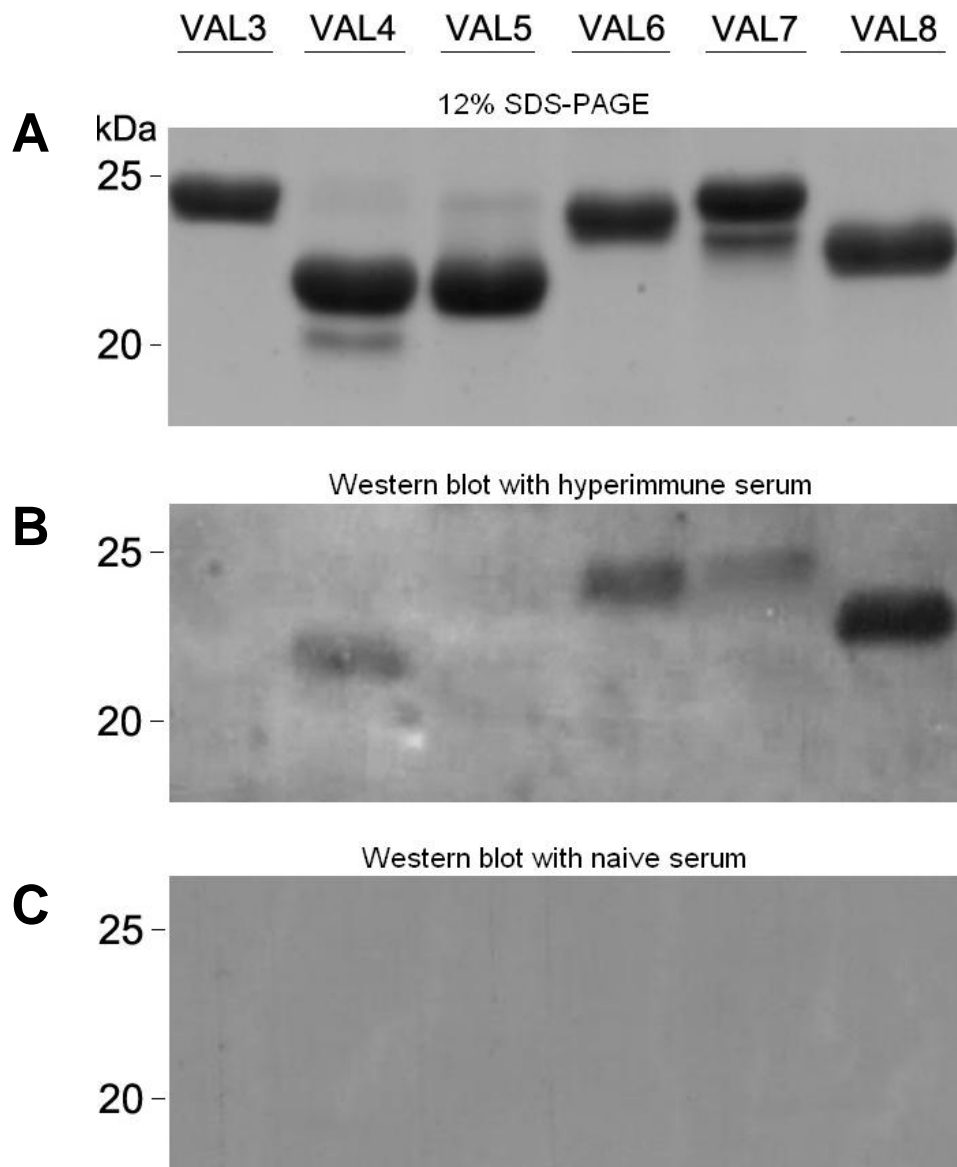
**Figure 5.4. Reactivity of A) Adult secreted proteins and B) L3A secreted proteins with anti-*Nb*VALs in Western blotting analysis.** Adult or L3A secreted proteins were resolved by 12% SDS-PAGE, blotted to nitrocellulose membranes and probed individually with anti-*Nb*VAL1, 3-8 and a pre-bleed serum (-ve) produced in mice, at a 1:400 dilution. Anti-mouse IgG-HRP secondary antibody was used (1:2000). The P lane shows the profile of proteins stained with DB71. Molecular weight markers are shown in kilodaltons. Open red squares indicate putative VAL proteins at their predicted sizes.

Recombinant *NbVAL3-8* were also tested for immune recognition by hyperimmune mouse serum (Fig. 5.5). *NbVAL4, 6, 7, and 8* were all recognised, with *NbVAL8* reacting most strongly, whereas *NbVAL3* and *NbVAL5* did not react (Fig. 5.5B). Naïve mouse serum showed no reactions to any of the recombinant proteins, showing that the binding is likely to be specific (Fig. 5.5C). This experiment also showed that antibodies produced against the natural native protein were capable of recognising the recombinant proteins. The immunogenicity exhibited by *NbVAL4, 6, 7 and 8* suggested that they may be suitable candidates for further vaccination experiments.

### **5.3. Expression testing of *NbVALs* in *E. coli* SHuffle cells**

Expression of the *NbVAL* proteins in *E. coli* BL21(DE3) cells previously resulted in only insoluble products, which is usually an indicator of incorrect folding. For functional analysis and vaccination studies, a structurally accurate protein which is as similar as possible to the native product should be produced. This is a considerable challenge as recombinant VALs are notoriously difficult to express in a soluble and stable form due to their high cysteine content. Attempts have been made to optimise their expression in BL21(DE3), but in none of the conditions were soluble proteins produced. Multiple attempts to express *NbVALs* in the yeast *Pichia pastoris* X-33 strain were also unsuccessful.

For want of a better system, expression was tested in *E. coli* SHuffle cells (New England Biolabs). SHuffle is a strain of *E. coli* which can promote protein folding through the constitutive expression of the disulphide bond isomerase DsbC. *NbVAL7* was chosen for the initial expression testing and optimisation, as it was one of the candidates for vaccination experiments. *NbVAL7* is secreted by both L3 and adults, and was shown to be immunogenic in natural infection.



**Figure 5.5. *NbVALs* are differentially reactive to hyperimmune serum.** A) Purified recombinant *NbVAL3-8* produced in the BL21(DE3) strain of *E. coli* resolved by 12% SDS-PAGE and visualised by staining with coomassie blue. B) Western blotting analysis of *NbVAL3-8* with hyperimmune serum at a dilution of 1:400. Anti-mouse IgG-HRP secondary antibody was used (1:2000). C) Negative control, reaction with pooled serum from naïve mice at a 1:400 dilution.

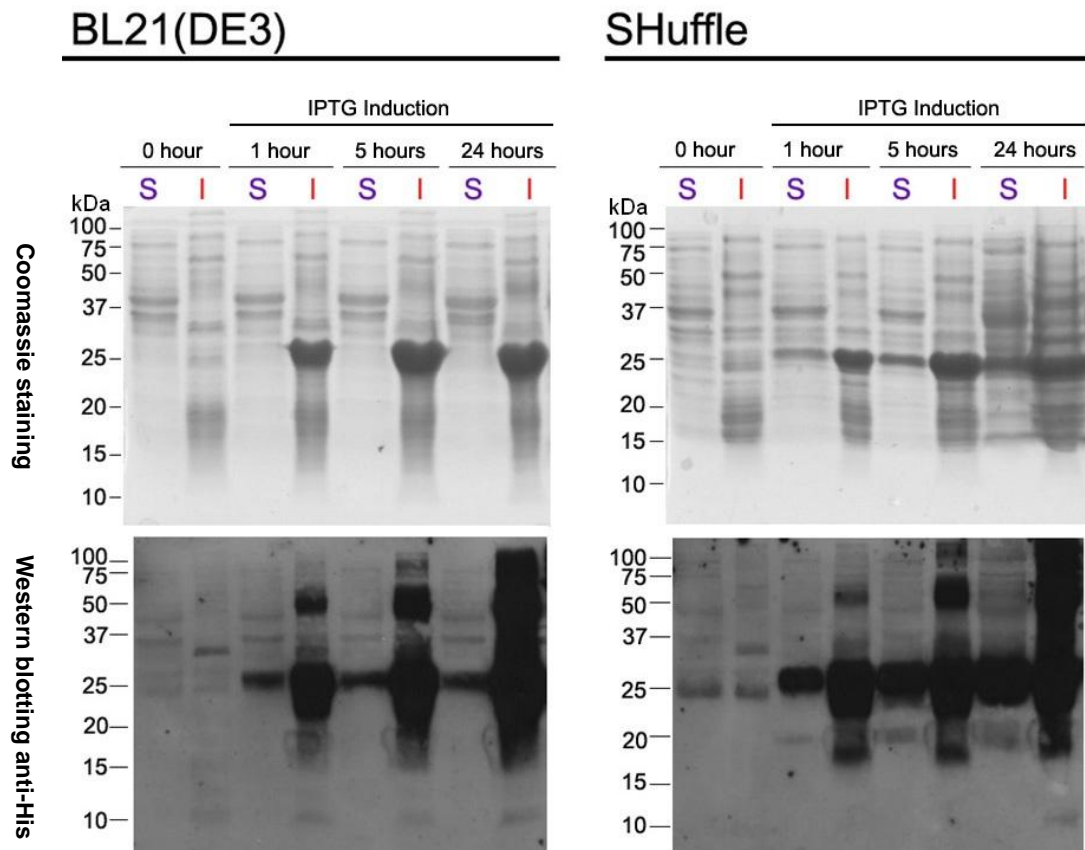
The pET29b-*NbVAL7* plasmid was purified and transformed into both BL21(DE3) and SHuffle competent cells for direct comparison. Expression tests at 30°C with IPTG induction showed that SHuffle cells consistently expressed a proportionately larger amount of soluble proteins than BL21(DE3) cells (Fig. 5.6). However, a large proportion of the proteins expressed in SHuffle cells still lay in the insoluble fraction.

It was further observed that some *NbVAL7* proteins were being expressed by SHuffle cells before IPTG induction (0 hour fractions, Fig. 5.6). This is indicative of ‘leaky’ expression, which I next aimed to exploit. Expression tests were performed again at 30°C, but without IPTG induction (Fig. 5.7). Under these conditions, BL21(DE3) cells produce virtually no recombinant proteins. In contrast, a large amount of mostly soluble *NbVAL7* protein was produced in SHuffle cells. It is probable that without IPTG induction, the *NbVAL7* protein was being expressed at a much slower rate, allowing more time for the protein to fold correctly in the cytosol, producing a soluble product.

With this rationale, the expression tests were repeated at 16°C, as a lower temperature also results in a slower expression of proteins. However, expression of soluble proteins was not particularly enhanced (Fig. 5.8). At the 24-hour timepoint, uninduced SHuffle cells produce more insoluble *NbVAL7* at 16°C than 30°C. SHuffle cells were found to grow very slowly at 16°C, and an incubation period of three days was needed before the culture was at the point where  $OD_{600nm} = 0.6$  (0 hour).

It was therefore determined that optimal expression of soluble *NbVAL7* could be carried out without IPTG induction at 30°C, and the cells could be harvested at 24 hours after  $OD_{600nm} = 0.6$ . Expression tests were carried out next for *NbVAL3-8* under these conditions, to analyse whether the other *NbVAL* variants could be expressed in soluble form.

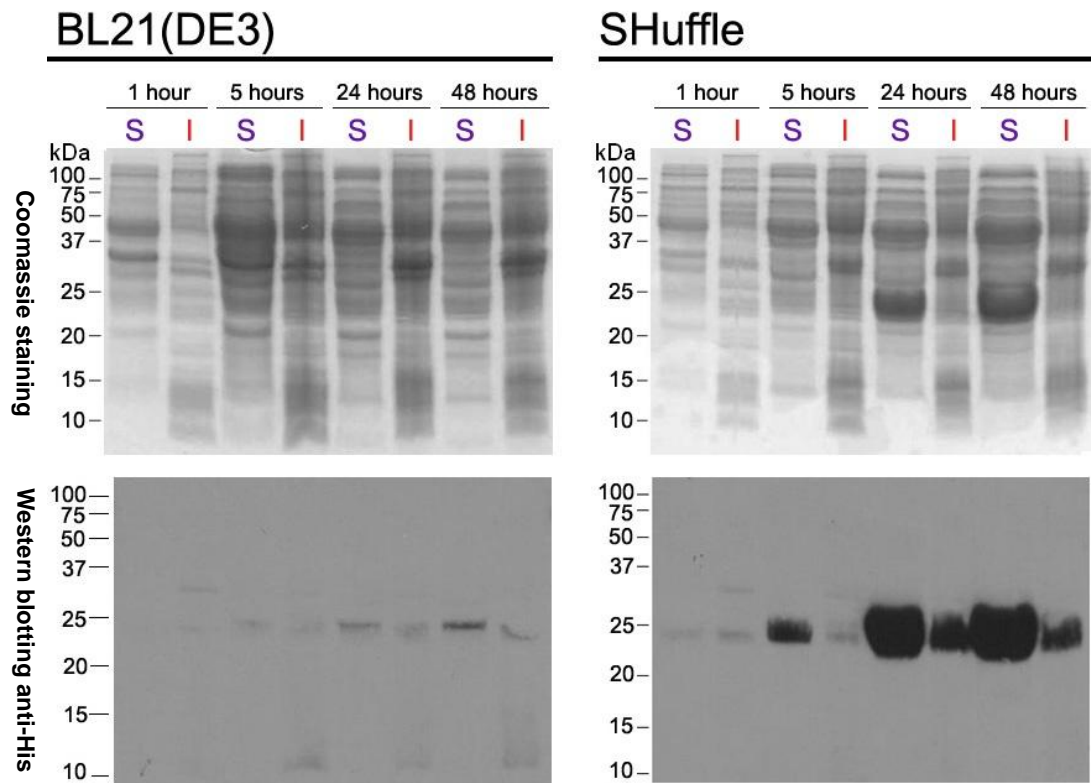
S: Soluble protein fraction  
I : Insoluble protein fraction



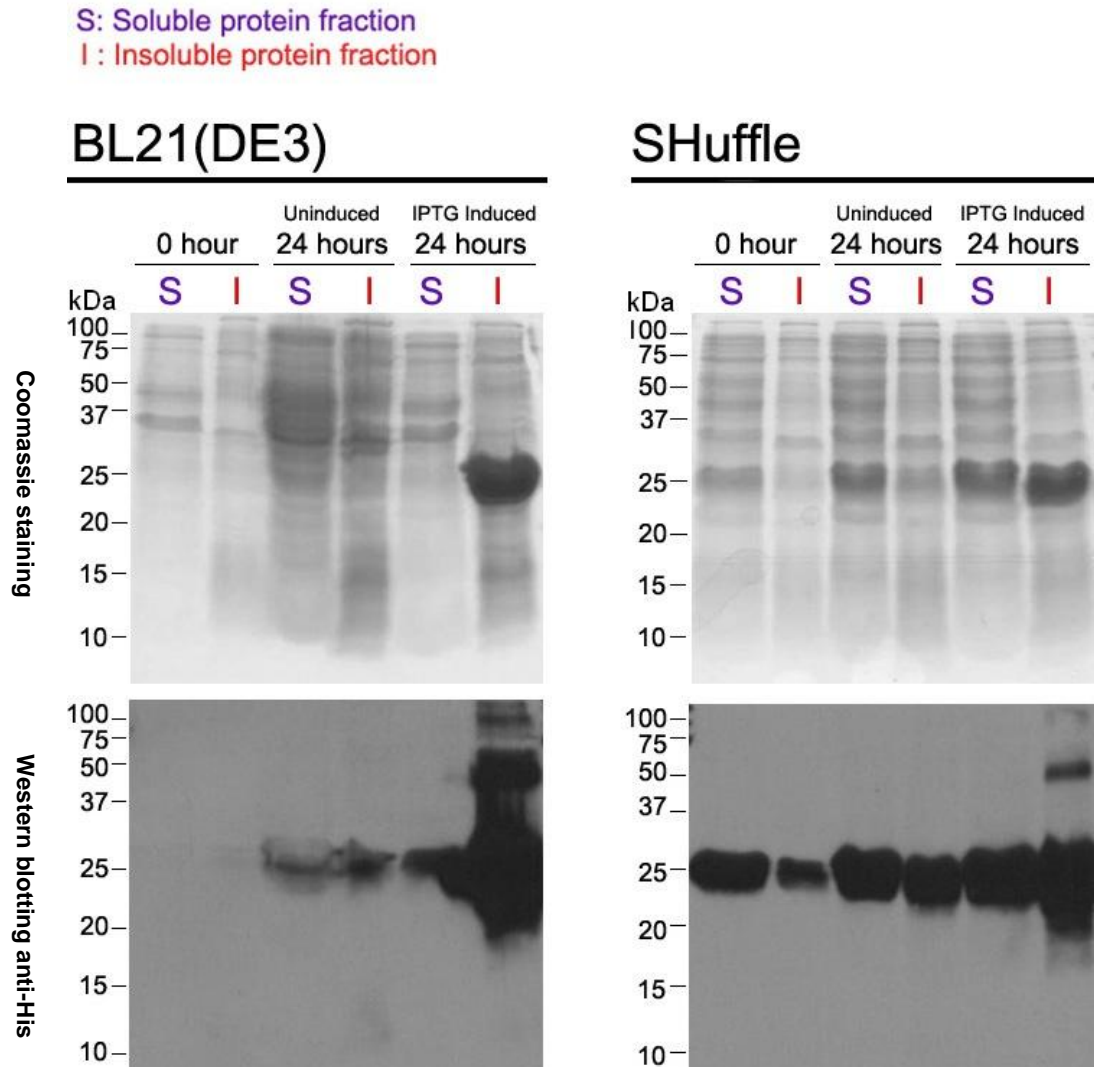
**Figure 5.6. Expression of *NbVAL7* in BL21(DE3) and *E. coli* SHuffle cells compared, at 30°C with IPTG induction.** Transformed cells were cultured until OD<sub>600</sub> reached 0.6 (0 hour), then induced with 1 mM IPTG. Cell aliquots were taken out at 1, 5 and 24 hours post-induction and separated into soluble and insoluble fractions, which were then resolved by 15% SDS-PAGE and reacted with anti-His using Western blotting analysis.



S: Soluble protein fraction  
I: Insoluble protein fraction



**Figure 5.7. Expression of *NbVAL7* in BL21(DE3) and *E. coli* SHuffle cells compared, at 30°C without IPTG induction.** Transformed cells were cultured until OD<sub>600</sub> reached 0.6. Cell aliquots were taken out at 1, 5, 24 and 48 hours post-induction and separated into soluble and insoluble fractions, which were then resolved by 15% SDS-PAGE and reacted with anti-His by Western blotting analysis.



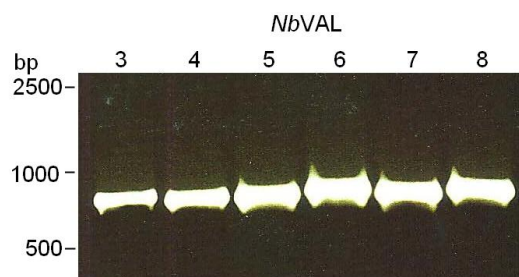
**Figure 5.8. Expression of *NbVAL7* in BL21(DE3) and *E. coli* SHuffle cells compared at 16°C.** Transformed cells were cultured until OD<sub>600</sub> reached 0.6 (0 hour). Each culture was then split into two, so that one continued to grow under the same conditions and the other was induced with 1 mM IPTG. Cell aliquots were taken out at the 24 hour time-point and separated into soluble and insoluble fractions, which were then resolved by 15% SDS-PAGE and reacted with anti-His using Western blotting analysis.

The pET29b plasmids containing *NbVAL3* to *NbVAL8* constructs were purified and verified for the correct insert by PCR with primers specific for each *NbVAL* variant (Fig. 5.9). All six plasmid preparations tested positive for the inserts and at the correct size at 650-700 base pairs (bp). Previous sequencing efforts had determined the inserts to be in frame and including a poly-histidine tag. The pET29b-*NbVAL* plasmids were transformed into SHuffle cells and expressed at 30°C without IPTG induction in small cultures for expression testing. However, soluble proteins could only be obtained for *NbVAL7*, whereas the expressed proteins of *NbVAL3*, 4, 5, 6 and 8 were all insoluble. Expression tests were repeated at 26°C, and soluble proteins were found to be expressed for *NbVAL7* and *NbVAL3*, and in very small amounts for *NbVAL4* and *NbVAL5* (Fig. 5.10).

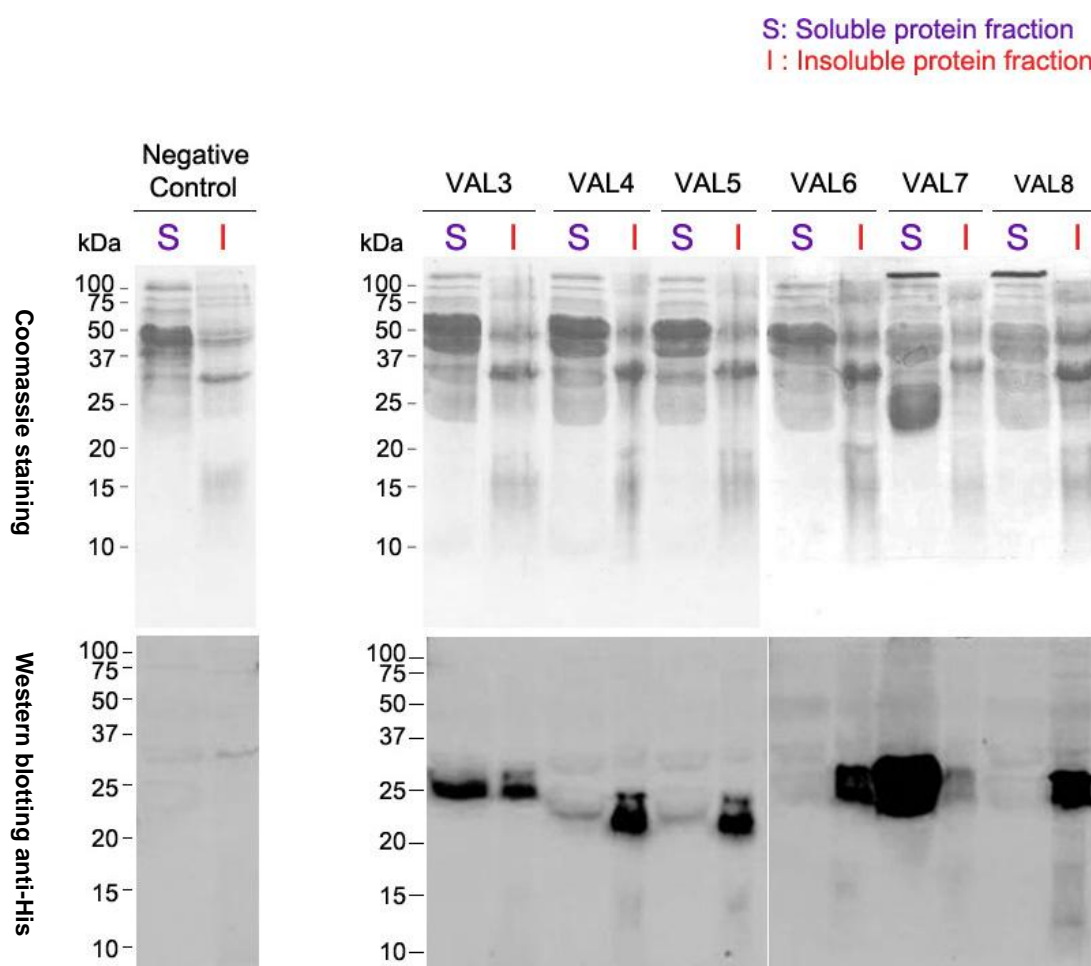
#### **5.4. Large scale expression and purification of *NbVALs* in *E. coli* SHuffle cells**

Half litre cultures each of *NbVAL3-8* were grown, and large amounts of soluble *NbVAL7* could be purified under native conditions (Fig. 5.11). Lesser amounts of soluble protein could be purified for *NbVAL4*, *NbVAL6* and *NbVAL3*. I decided to proceed with *NbVAL7* and *NbVAL4*, a double domain VAL expressed by both L3 and adults and a single domain VAL exclusive to the adult stage, respectively.

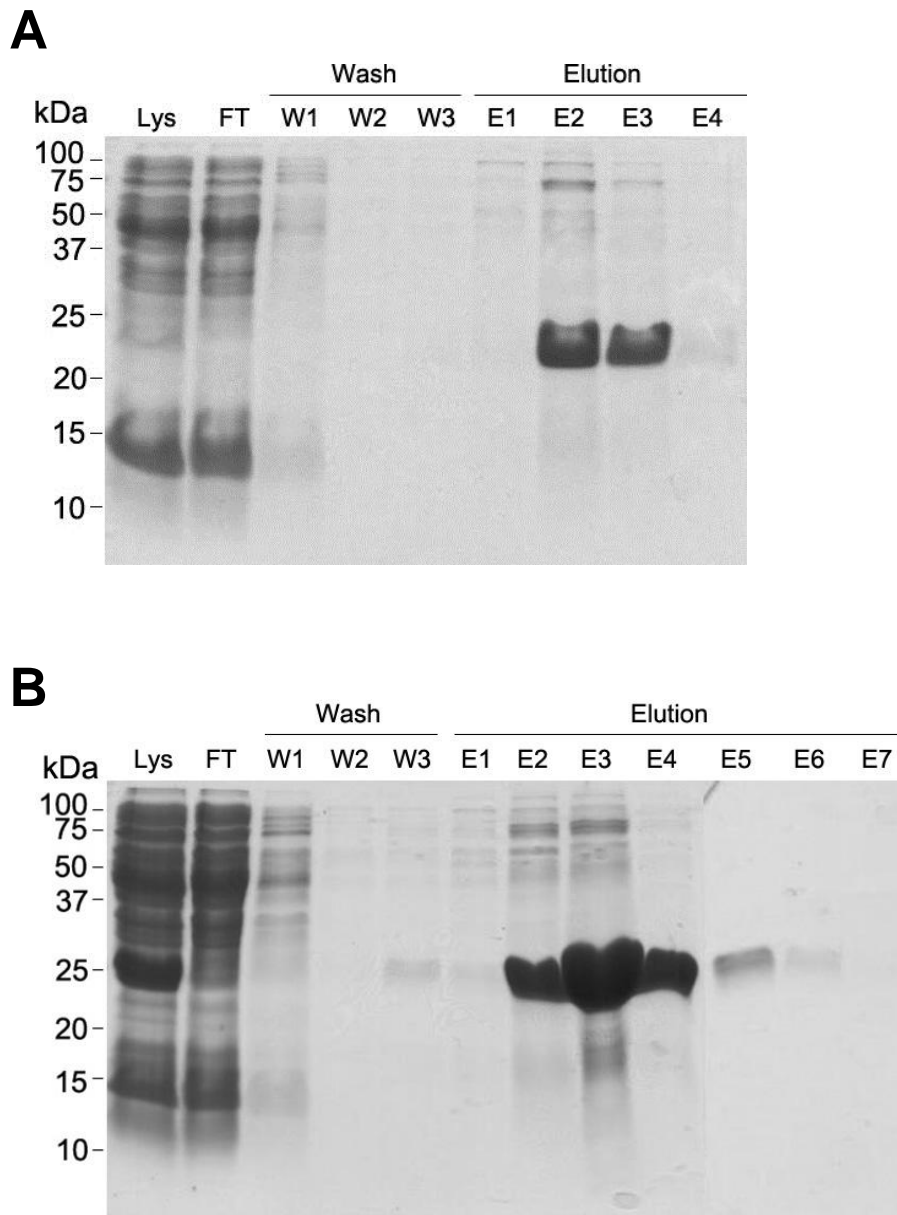
Eluted fractions of *NbVAL4* and *NbVAL7* were individually dialysed in PBS. Both proteins were stable throughout dialysis with no precipitates formed. The yields for *NbVAL4* and *NbVAL7* were 0.72 mg and 8.8 mg of recombinant protein per half litre culture respectively. The elution fractions with higher purity, E3 for *NbVAL4*, and elution fractions E4, E5 and E6 for *NbVAL7* (pooled) were used in further experiments (Fig. 5.11).



**Figure 5.9. PCR products of *NbVAL3-8* from purified pET29b plasmids containing *NbVAL3* to *NbVAL8* constructs.** PCR was carried out using primers specific for each individual *NbVAL* variant, and resolved by agarose gel electrophoresis (0.8% w/v). PCR products were as expected at 650-700 base pairs (bp).



**Figure 5.10. Expression of *NbVAL3-8* in *E. coli* SHuffle cells at 26°C.** Transformed cells were grown until OD<sub>600</sub> reached 0.6, then cultured for another 24 hours. Cell aliquots were sampled at the 24 hour time-point and separated into soluble and insoluble fractions, which were then resolved by 15% SDS-PAGE and reacted with anti-His using Western blotting analysis. A negative control was included with untransformed competent SHuffle cells.



**Figure 5.11. Purification of A) *NbVAL4* and B) *NbVAL7* produced in *E. coli* SHuffle under native conditions.** Polyhistidine-tagged *NbVAL4* and *NbVAL7* were purified from cleared cell lysates (Lys) from 500 ml of culture using nickel affinity chromatography. After the flow-through (FT) fraction was collected, columns were washed with 20 mM imidazole (W1 and W2) and 40 mM imidazole (W3). Recombinant proteins were eluted with 250 mM imidazole in 0.5 ml aliquots (E1-E7).

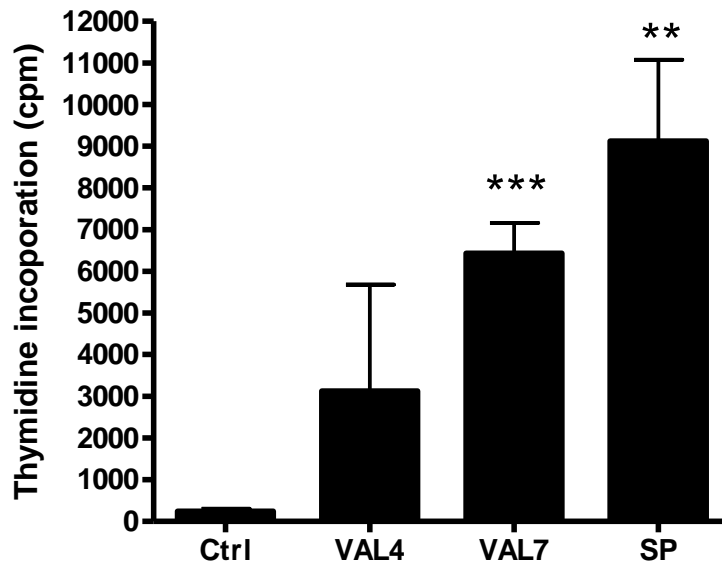
## 5.5. Immunogenicity of *NbVALs*

Cellular and humoral responses of multiply infected (hyperimmune) mice to *NbVAL4* and *NbVAL7* were tested to evaluate their immunogenicity. LPS was removed from protein preparations prior to experiments.

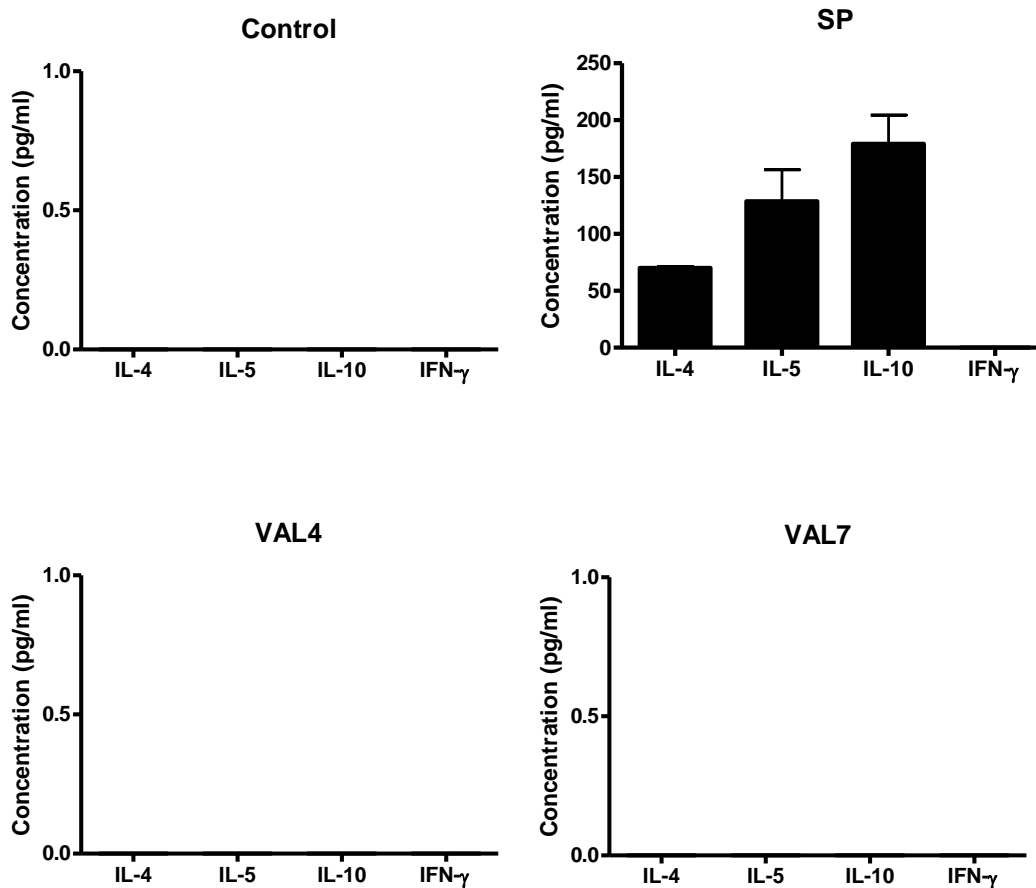
Splenocytes were isolated from mice infected four times with *N. brasiliensis* and tested for cellular proliferation in response to *NbVAL4* and *NbVAL7* (Fig. 5.12). It was found that significant proliferation was stimulated by *NbVAL7* but not by *NbVAL4*. *N. brasiliensis* adult secreted products (SP) were used as a positive control, which stimulated a higher level of proliferation than *NbVAL7*. This shows that like *N. brasiliensis* SP, *NbVAL7* can stimulate a cellular immune response in the spleen.

Splenocytes from infected mice were also assayed for cytokine release. As expected, stimulation with *N. brasiliensis* secreted products resulted in secretion of IL-4, IL-5, IL-10 but no IFN- $\gamma$ , a typical cytokine profile of a Th2-biased response elicited by parasitic nematode infection (Fig. 5.13). However, no detectable secretion of these cytokines could be found on stimulation with *NbVAL4* or *NbVAL7*.

Serum samples were also collected from infected mice to assay for the humoral response to *NbVAL4* and *NbVAL7*. Sera from five mice were pooled and titrated to test for levels of IgG1, IgG2a, IgG2b, IgG3, IgA and IgE specific for *NbVAL4* or *NbVAL7*. It was found that the overall reaction to *NbVAL7* was stronger than *NbVAL4* (Fig. 5.14). Individual samples were then tested at a specific dilution for each immunoglobulin class or subclass to account for individual variation (Fig. 5.15). The humoral response to *NbVAL4* and *NbVAL7* followed a similar trend; of the IgG subclasses, only IgG1 levels were significantly higher in infected mice compared to naïve controls, with no detectable differences in IgG2a, indicative of a Th2-biased response. Antigen-specific IgA was also produced, a response typical of gastrointestinal helminth infections. Antigen-specific IgE was detected, suggesting that *N. brasiliensis* VAL proteins had the potential to induce immediate-type hypersensitivity.

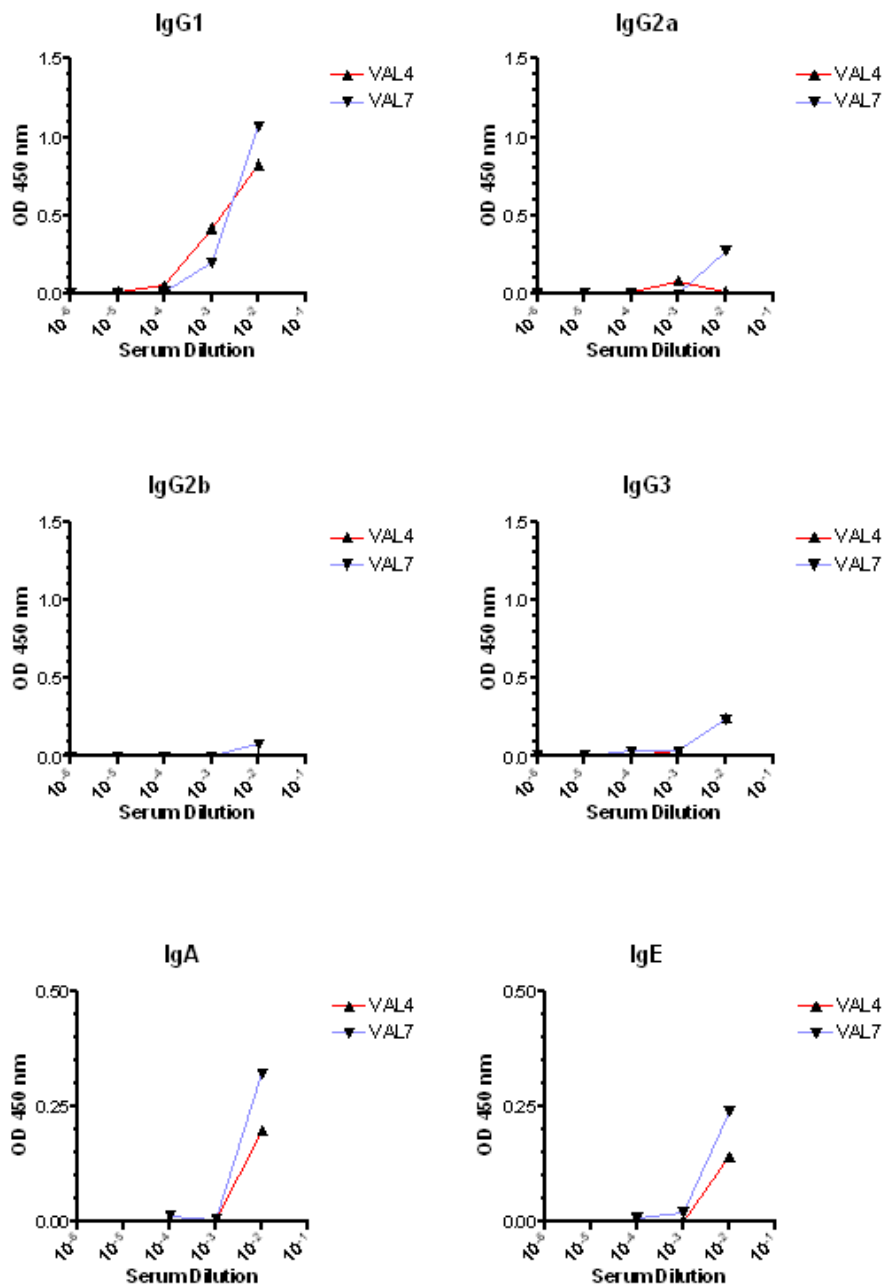


**Figure 5.12. Splenocytes from multiply infected mice proliferate in response to *NbVAL7* and adult secreted proteins.** Splenocytes were harvested from mice infected 4 times with *N. brasiliensis*, 1 week after the final infection. Cells were cultured with adult secreted products (SP), *NbVAL4* or *NbVAL7* at 10  $\mu\text{g/ml}$ , or in medium alone (Ctrl) for 60 hours, then for another 16 hours with  $^3\text{H}$ -thymidine added at 1  $\mu\text{Ci}$  per well. Background values from splenocytes of naïve mice treated identically were subtracted. Results represent mean counts per minute + SEM ( $n = 5$ ) from splenocytes of 5 individual mice assayed in quadruplicate. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ , relative to control.



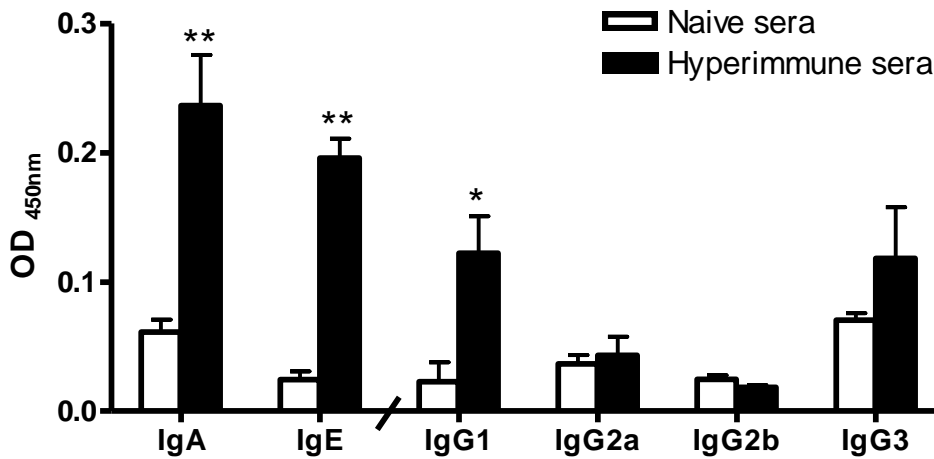
**Figure 5.13. Cytokine release from splenocytes of multiply infected mice.** Splenocytes were harvested from mice infected 4 times with *N. brasiliensis*, 1 week after the final infection. Cells were cultured with adult secreted products (SP), *NbVAL4* or *NbVAL7* at 10  $\mu$ g/ml, or in medium alone (Ctrl) for 60 hours, when the medium was collected for cytokine measurement by ELISA for IL-4, IL-5, IL-10 and IFN- $\gamma$ . Background values from splenocytes of naïve mice treated identically were subtracted. Results represent mean values + SEM ( $n = 5$ ) from splenocytes of 5 individual mice assayed in quadruplicate.



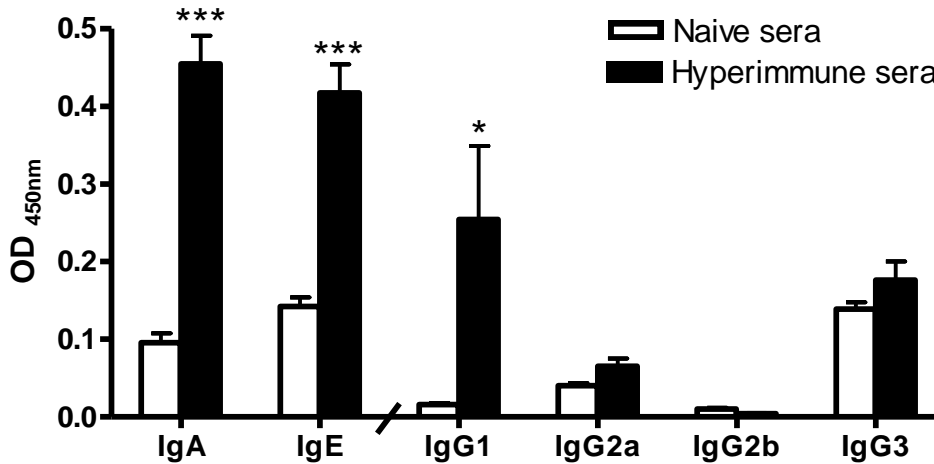


**Figure 5.14. Antibody responses to *NbVAL4* and *NbVAL7* in infected mice (titration).** Plates were coated with *NbVAL4* or *NbVAL7* at 5  $\mu\text{g/ml}$  and reacted with hyperimmune serum pooled from 5 multiply infected mice, diluted to the indicated concentrations. HRP-conjugated secondary antibodies to immunoglobulin isotypes were used, and background values were subtracted. Samples were assayed in triplicate.

### VAL4-specific antibody responses



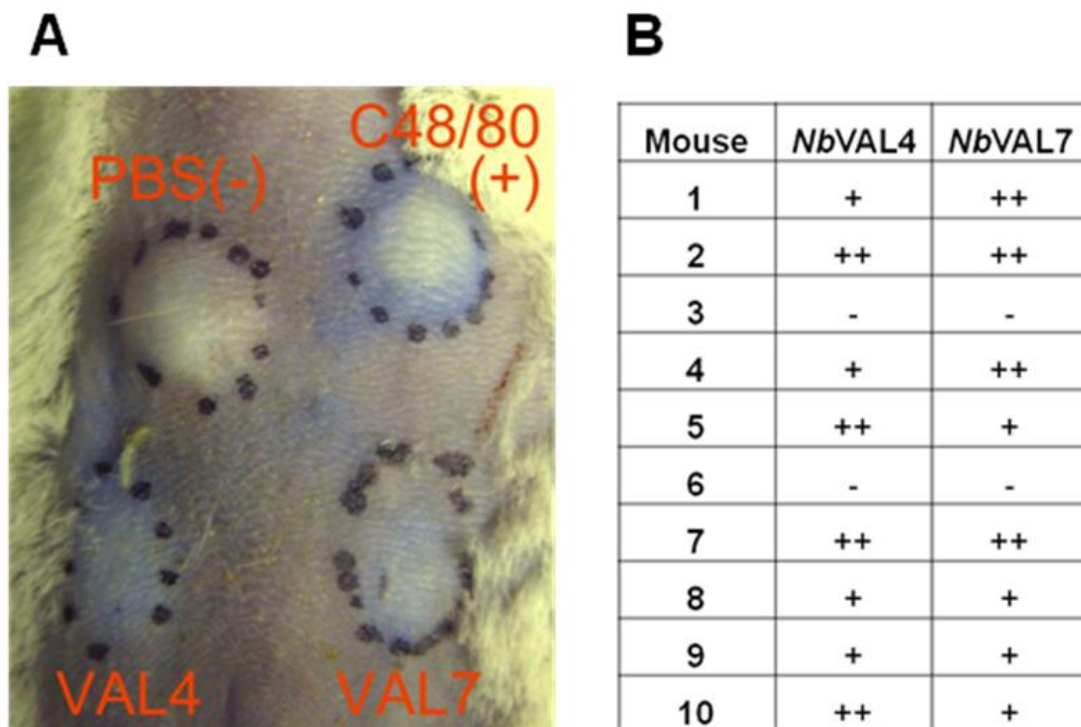
### VAL7-specific antibody responses



**Figure 5.15. Antibody responses to *NbVAL4* and *NbVAL7* in infected mice.** Serum samples were used at a 1:100 dilution for IgA and IgE detection, and at a 1:1000 dilution for all IgG subclasses for direct comparison. Results represent mean values + SEM ( $n = 5$ ) from immune sera of 5 multiply-infected mice assayed in triplicates. \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ , relative to naïve serum control.

## 5.6. Type 1 hypersensitivity induced by *NbVAL4* and *NbVAL7*

To test if exposure to *N. brasiliensis* infection made mice susceptible to immediate (type 1) hypersensitivity reactions on subsequent challenge with VALs, multiply infected mice were subjected to intradermal challenge with *NbVAL4* and *NbVAL7*, followed by monitoring local mast cell degranulation using Evans blue extravasation as a read-out (Fig. 5.16A). The mast cell activating compound C48/80 was used as a positive control, which induced a local area of extravasation in every mouse tested. PBS was used as a negative control, which did not elicit reactions in any of the mice. Challenge with *NbVAL4* and *NbVAL7* resulted in clear positive reactions (++) in 4 out of 10 mice, whereas 2 out of 10 did not react to either antigen (Fig. 5.16B). These results strongly suggest that the *NbVAL4* and *NbVAL7* antigens are potential inducers of type I hypersensitivity.



**Figure 5.16. *NbVAL4* and *NbVAL7* induce Type I hypersensitivity in infected mice.** Passive cutaneous anaphylaxis reactions were induced by *NbVAL4* and 7, using Evans blue extravasation as a read-out. Mice infected 4x were challenged with 1  $\mu$ g of *NbVAL4* and *NbVAL7*, 500 ng of mast cell activating compound C48/80 (positive control), and PBS (negative control). A) Photograph of skin reactivity taken 20 minutes after antigen challenge. B) Strength of reaction in individual mice, with + designated as weak and ++ as strong reactivity. A non-significant difference between results obtained for *NbVAL4* and *NbVAL7* was determined using the Mann-Whitney rank sum test ( $P < 0.05$ ) on the Graphpad Prism software.

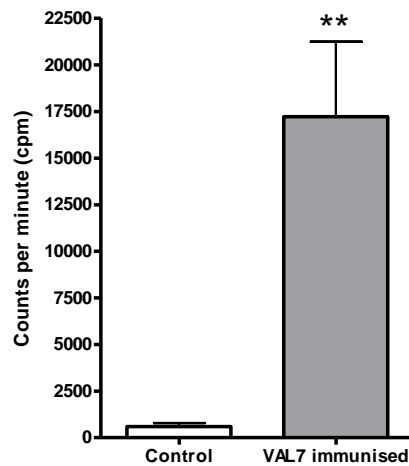
### 5.7. Immunisation with *NbVAL7*

*NbVAL7* was chosen for the immunisation experiments because, like the *Na-ASP-2* used in human clinical trials, it is secreted by L3. It also showed higher immunogenicity than *NbVAL4* in terms of stimulating cellular proliferation and antibody responses.

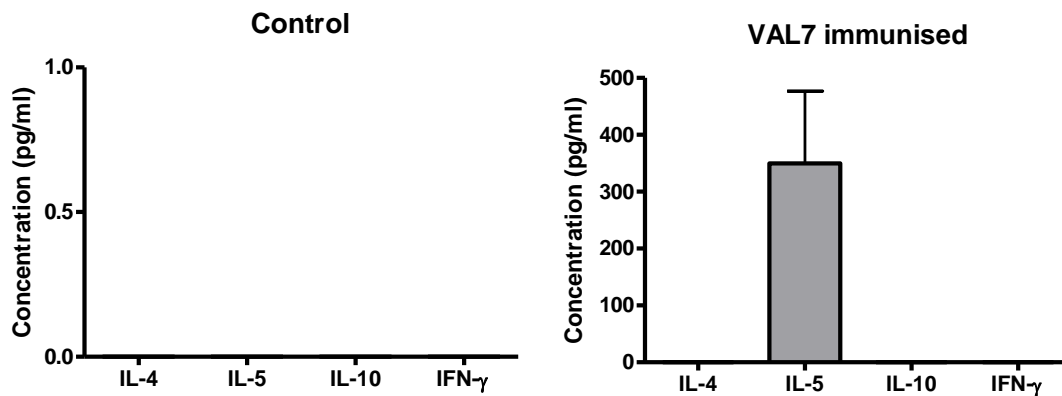
Female BALB/c mice were immunised with 25 µg of *NbVAL7* precipitated with potassium aluminium sulphate (alum) as adjuvant, and thereafter were each boosted twice with 15 µg of *NbVAL7*-alum at monthly intervals. A control group of mice matched to the test subjects were immunised and boosted with an equivalent amount of PBS-alum at the same time points. One week after the final boost, mice from both groups were tested for their immune response to *NbVAL7* and challenged with 500 *N. brasiliensis* L3.

Cellular immune responses were tested one week after the final boost. Splenocytes harvested from immunised mice showed a high proliferative response (Fig. 5.17), and secreted IL-5 on stimulation with *NbVAL7* (Fig. 5.18). The humoral response was assessed by antigen-specific ELISA with sera collected from immunised mice. The serum samples were first pooled and titrated (Fig. 5.19). Individual samples were then each tested at the optimal dilution for each specific immunoglobulin class or sub-class (Fig. 5.20, open bars, 'pre-challenge with L3' group). Immunisation stimulated production of *NbVAL7*-specific IgG1, IgG2a, IgG2b, but not IgG3. IgG1 responses were significantly and consistently higher than IgG2a, perhaps indicative of a slightly Th2-skewed response. It is particularly noteworthy that, even before challenge with *N. brasiliensis* L3, immunisation resulted in production of *NbVAL7*-specific IgE, demonstrating the allergenic potential of the VAL antigen.

Serum samples were also collected post-challenge with L3. There was a slight trend for boosting of antigen-specific IgG2a, IgG2b and IgE by challenge infection (Fig. 5.19), but in no case was this statistically significant (Fig. 5.20, black bars, 7 days post-challenge group).

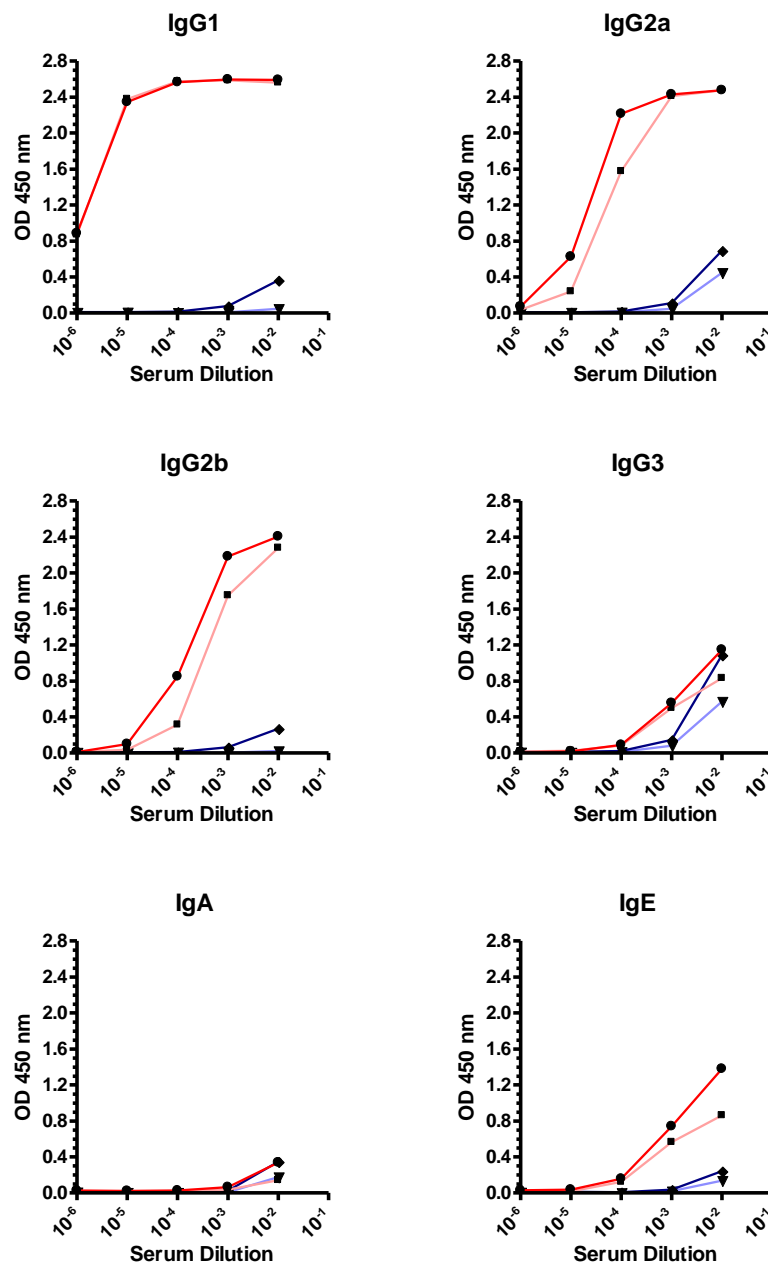


**Figure 5.17. Splenocytes from *NbVAL7* immunised mice proliferate in response to recombinant *NbVAL7*.** Splenocytes were harvested from mice immunised with *NbVAL7* conjugated to alum, 1 week after the final boost. Cells were cultured with *NbVAL7* at 10 µg/ml, or in medium alone (Ctrl) for 60 hours, then for another 16 hours with <sup>3</sup>H-thymidine added at 1 µCi per well. Background values from identically treated splenocytes of alum-immunised mice were subtracted. Results represent mean counts per minute + SEM from splenocytes of 5 individual mice assayed in quadruplicate. \*\*  $P < 0.01$ , relative to control.

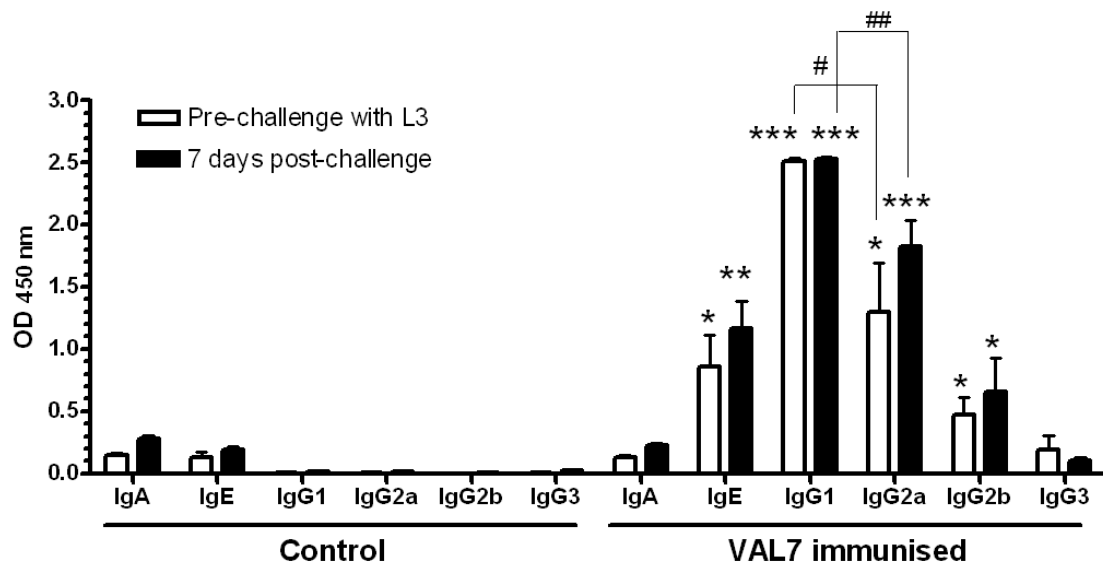


**Figure 5.18. Cytokine release from splenocytes of *NbVAL7* immunised mice.** Splenocytes were harvested from mice immunised with *NbVAL7* conjugated to alum, 1 week after the final boost. Cells were cultured with *NbVAL7* at 10 µg/ml, or in medium alone (Ctrl) for 60 hours, when the medium is collected for cytokine measurement by ELISA for IL-4, IL-5, IL-10 and IFN-γ. Background values from identically treated splenocytes of alum-immunised mice were subtracted. Results represent mean values + SEM from splenocytes of 5 individual mice assayed in quadruplicate.

- ▼ Control
- ◆ Control, L3 challenged
- VAL7 immunised
- VAL7 immunised, L3 challenged



**Figure 5.19. Titration of antibody responses to *NbVAL-7* in immunised and naive mice, pre- and post-challenge with infective larvae.** Plates were coated with *NbVAL7* at 5 µg/ml and reacted with serum pooled from 5 immunised mice, diluted to the indicated concentrations. HRP-conjugated secondary antibodies to immunoglobulin isotypes were used. Samples were assayed in triplicate.

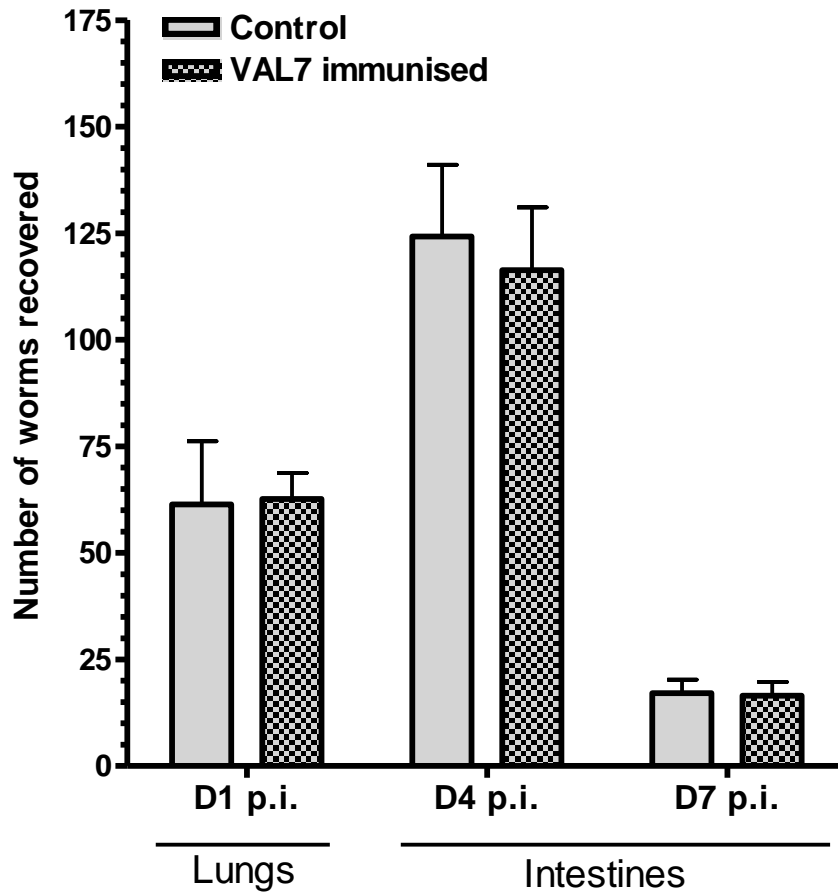


**Figure 5.20. Antibody responses in *NbVAL7* immunised mice.** Mice were immunised with *NbVAL7* conjugated with alum, or alum alone (control). Sera were collected before and after challenge with *N. brasiliensis* L3 from 5 individual mice per group, and assayed by ELISA in triplicate. Sera were diluted 1:100 for IgA and IgE measurement, and 1:5000 for all IgG subclasses for direct comparison. Results are presented as mean levels of *NbVAL7*-specific immunoglobulin +SEM ( $n = 5$ ). \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , relative to control. Hash signs indicate a significant difference between levels of the IgG1 and IgG2a isotypes; ## $P < 0.01$ , # $P < 0.05$ .

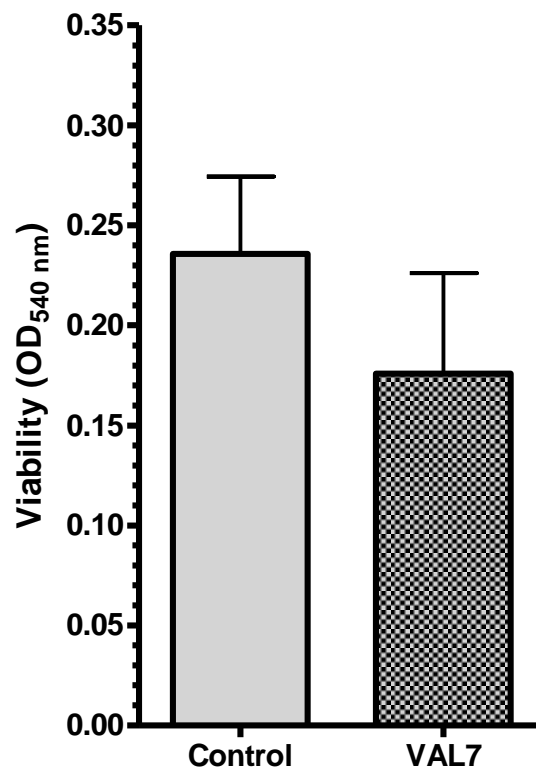


Immunisation with *NbVAL7* was thus successful in terms of producing strong antigen-specific cellular and humoral responses. To assess whether these conferred any protective effect against *N. brasiliensis* infection, parasite recovery was quantified from control and immunised mice post-challenge. Worms were recovered from the lungs of mice on day 1 post-infection (D1 p.i.), and from the intestines on D4 and D7 p.i. (Fig. 5.21). Worms collected on D1, D4, and D7 consist of L4, young adults and mature adults respectively, and reflect the ability to infect, migrate and persist in different anatomical compartments. It was found that parasite recovery numbers were similar between the control and *NbVAL7* immunised groups at all three time points. The numbers on D1 p.i. provide a snapshot of L4 transiting through the lungs at that particular timepoint, consisting of  $12.3 \pm 3.0\%$  and  $12.5 \pm 1.2\%$  of the initial infection dosage of 500 L3 for the control and immunised group respectively. The percentage establishment of adults on D4 p.i. was  $24.9 \pm 3.4\%$  for the control group, and  $23.3 \pm 3.0\%$  for the immunised group. Most worms were expelled from the mice by D7 p.i., with  $3.4 \pm 0.6\%$  and  $3.3 \pm 0.7\%$  remaining for the control and immunised groups respectively. These results show that no protective effects were conferred in terms of total numbers of parasites recovered at different stages of the life cycle. Worms isolated on D4 p.i. were also tested for viability by the MTT assay. This did not differ between the groups (Fig. 5.22), suggesting that immunisation with *NbVAL7* did not compromise parasite fitness.

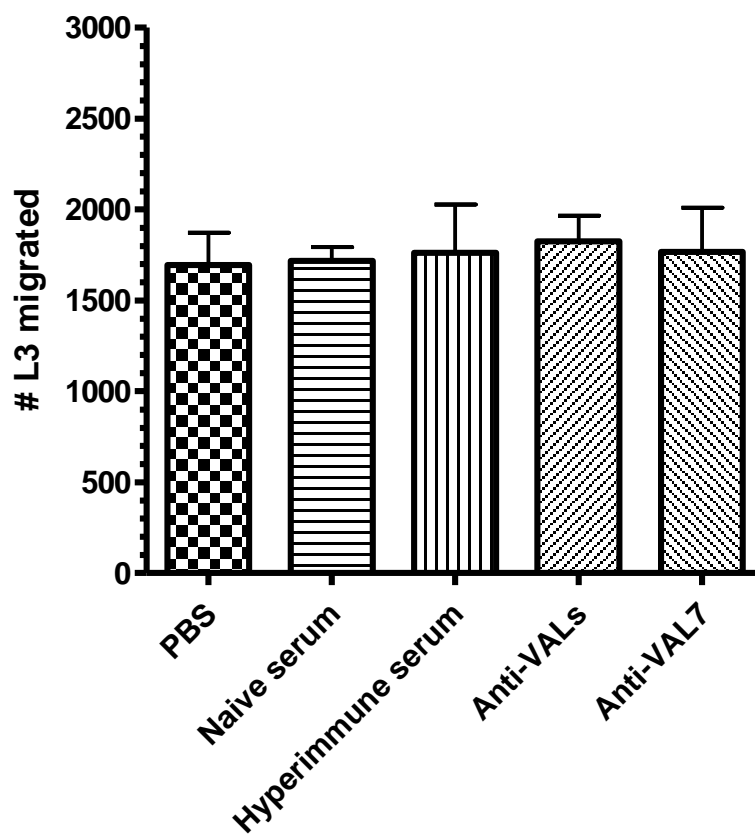
Since antisera to *Na-ASP-2* has been shown to inhibit penetration of dog skin by L3 *in vitro* (Bethony *et al.*, 2005), I replicated this experiment with antisera to the *NbVALs*. Pre-incubation of *N. brasiliensis* L3 with anti-*NbVAL7* collected from the above immunisation experiments did not affect their migration into rat skin, as compared to those incubated in naïve serum or PBS (Fig. 5.23). Pre-incubation with a mixture of antisera to *NbVAL1* and *NbVAL3-8* had no effect either. Pre-incubation with hyperimmune serum also no effect on L3 penetration, suggesting that the skin is an unlikely site of effective immune action to eliminate *N. brasiliensis*, at least when considered in terms of antibody-mediated mechanisms.



**Figure 5.21. Immunisation with *NbVAL7* does not confer protection against infection with *N. brasiliensis*.** Mice were immunised with *NbVAL7* conjugated with alum, or alum alone (control). Both groups were challenged with 500 *N. brasiliensis* L3 one week after the final boost. Mice were sacrificed on day 1, day 4 and day 7 post-infection (p.i.) in groups of 8 per condition. Worms were recovered from the lungs on day 1 p.i., and from the intestines on day 4 and day 7 p.i. Results are presented as the mean number of worms recovered + SEM ( $n = 8$ ).



**Figure 5.22. Immunisation of mice with VAL7 has no effect on parasite viability at the intestinal stage.** Viability of worms recovered on day 4 p.i. was measured by the MTT assay, with 50 worms per group, measured in triplicate. Results are presented as the mean absorbance value at 540 nm +SEM ( $n = 3$ ).



**Figure 5.23. Penetration of rat skin by *N. brasiliensis* L3 is not affected by immune serum or antibodies to *Nb*VALs.** *N. brasiliensis* L3 were incubated with PBS, naïve serum, immune serum, mixed anti-*Nb*VALs or anti-*Nb*VAL7 for 1 hour prior to the skin penetration test, in which L3 were loaded onto a testing chamber sandwiching a piece of intact rat skin and left to migrate for 30 minutes. Results are presented as the mean number + SEM ( $n = 3$ ) of migrated worms, assayed in triplicate.

## 5.8. Discussion

Discovery of the eight variants of VALs in *N. brasiliensis* provided an opportunity to improve our understanding of these proteins under experimentally controlled methods, the tools of which are limited for the human hookworm *N. americanus*. As *Na*-ASP-2 was selected as a major candidate by the Human Hookworm Vaccine Initiative (HHVI), it is informative to collect data in various systems for a broader evaluation of the potential of VALs for immunoprophylaxis of nematode infections in general. These are extremely important in light of the decreasing effectiveness of current anthelmintic treatments. The success of vaccines hinges on the following criteria: viable production methods, ability to stimulate protective immunity, and safety of administration. These themes will be the main topics of discussion.

### *Expression of VAL proteins*

While isolating natural products from the parasite itself would generate the target protein in its most native form, in practice nematodes secrete too little proteins for this procedure to be applicable, and downstream purification is not always a viable option. This is why an expression system must be employed to produce the target protein as a recombinant. There are high numbers of disulphide bridges in VAL proteins - it was predicted that there are 4 disulphide bonds in each of the single domain *Nb*VALs (1, 4, 5, 6), 8 bonds for *Nb*VAL2, 7 and 8, and 10 bonds for *Nb*VAL3 (Huang, 2010). Therefore, achieving correct folding in recombinants is challenging. Although all the information for a protein's ultimate conformation is encoded in its primary sequence, the actual folding process may become erratic once it is taken out of its native environment. In the eukaryotic cell, most disulphide bonds found in secreted proteins are formed in the oxidative environment of the rough endoplasmic reticulum after protein translation and translocation. Prokaryotic cells have no such compartments, and recombinant proteins produced in bacterial expression systems remain in the reducing environment of the cytosol, which is un conducive to protein folding. Also, bacterial systems are unable to provide the post-translational modifications and/or suitable molecular machinery and chaperones which some eukaryotic proteins require to achieve accurate and stable folding. Moreover, the use of strong promoters and

inducers to activate overexpression of the target recombinant often results in high concentrations of folding intermediates with exposed sulphhydryl (-SH) groups which may randomly interact with each other in an intra- or inter-molecular fashion in subsequent steps, making it even more difficult to produce recombinants which resemble the native structure (Sorensen and Mortensen, 2005). Unfolded or misfolded proteins have a tendency to form into insoluble aggregates called inclusion bodies in the bacterial cytosol. These inclusion bodies are usually heavily contaminated with other bacterial constituents, which are frequently not possible to eliminate completely, restricting the downstream applications of the recombinant proteins, particularly in cellular immunology and vaccination studies.

The *E. coli* SHuffle strain and the expression conditions used for the *NbVALs* were chosen with these considerations in mind. SHuffle cells were engineered to optimise efficient folding of proteins with high cysteine content by harnessing the constitutive expression of the disulphide bond isomerase DsbC, which catalyses the formation and reshuffling of disulphide bonds in the bacterial cytosol (Bessette *et al.*, 1999). DsbC also acts as a folding chaperone, which directs and stabilises the folded structure (Chen *et al.*, 1999). A lowering of the cultivation temperature is known to discourage hydrophobic interactions which contribute to aggregation reactions, and also results in a slower rate of protein synthesis which reduces the system's metabolic burden (Sorensen and Mortensen, 2005). This is also achieved by not using any inducers to stimulate overexpression, which is favourable because the recombinant proteins have more time to fold according to the conformational preferences of the nascent polypeptide chain, resulting in the alignment of the proper cysteine residues for disulphide bond formation. A slower rate of synthesis also means that there would be fewer interactions between molecules with exposed unoxidised -SH groups, minimising chain reactions of protein-protein aggregates.

Using the *E. coli* SHuffle system under these conditions, large amounts of soluble *NbVAL7* were produced by growing cells at 26°C without induction. It is critical for the expressed product to be soluble, because (1) there is higher fidelity in its correct folding, which would make it functionally and immunologically closer to the native protein, and (2) it is more suitable for most methods of experimentation (e.g. ELISA and cellular stimulation). The SHuffle system also enabled the expression of smaller

amounts of soluble *NbVAL4*, 6 and 3, which was still an improvement to the BL21(DE3) system which produced only insoluble proteins. It was somewhat surprising that proteins with such similar sequences should behave so differently, especially when the position of their cysteine residues, and thus presumably the formation of disulphide bonds, were also considerably conserved. It seems that further optimisation of expression of the *NbVAL* proteins is needed, which may possibly result in different conditions for each variant. In future work to express these proteins in soluble form, the following options may be considered.

Bacterial expression systems have remained a choice method for recombinant protein synthesis due to their utilisation of inexpensive carbon sources, simple and rapid growth, ability to withstand high density cultivation and simple process scale-up. Strategies that have been employed to express cysteine-rich proteins include export of the recombinant protein to the oxidising environment of the periplasmic space by the inclusion of an N-terminal translocation sequence, where the formation of disulphide bonds is energetically favourable and aided by multiple molecular chaperones and disulphide isomerases (Baneyx and Mujacic, 2004). Others have utilised similar concepts as the *E. coli* SHuffle strain by genetically engineering the cell cytoplasm into an environment conducive to protein folding. For example, the Origami and Rosetta gami (Novagen) strains of *E. coli*, a preferred choice of structural biologists, are cells with disabled thioredoxin reductase (TrxB) and glutathione reductase (gor). TrxB and gor are enzymes which actively keep cysteines in the *E. coli* cytoplasm in a reduced state, and their elimination was found to greatly enhance protein folding in the bacterial cytoplasm (Derman *et al.*, 1993). If all fails, refolding protocols may be considered. These generally involve the isolation and resolubilisation of inclusion bodies, followed by *in vitro* renaturation under conditions which favour protein folding and disulphide bond formation. These methods can however be associated with low recovery yields, and the resolubilisation process may compromise the integrity of the refolded proteins (Verma *et al.*, 1998). The optimal refolding conditions are also often different for different proteins, perhaps making it a more expensive option than optimising conditions to produce soluble proteins.

Bacterial systems are however still lacking in post-translational processing such as glycosylation, which may be important for protein structure and function. Multiple N-

linked glycosylation sites were predicted in *NbVAL2*, 3 and 8, although they do not show conserved patterns. Interestingly, O-linked glycosylation sites were predicted in the cysteine-rich / hinge region of *NbVAL2*, 3, 7 and 5 which appear to be positionally similar (Fig. 5.3). Moreover, multiple O-linked glycosylation sites have also been found in the hinge region of *Hp-VAL-1* of *H. polygyrus* (Murray *et al.*, 2010). Structural modelling showed that the hinge region lies apart from the SCP domains of the molecule like *Na-ASP-2*, suggesting that it is exposed to its surroundings and may be capable of interacting with other molecules. This could be of functional significance, as it is possible that the hinge region is more than just a linker connecting the two domains (in the case of double domain VALs) or a residual legacy from divergent evolution (for single-domain VALs). It is known that nematodes secrete many glycoconjugates including glycoproteins, mucins, C-type lectins and galactins (Dzik, 2006). In fact, the NIF protein, a VAL homologue purified from *H. contortus* and *A. caninum*, was found to be heavily glycosylated – the sugar moiety of *AcNIF* accounts for 40% of its molecular weight (Anbu and Joshi, 2008; Moyle *et al.*, 1994). Considering that carbohydrate moieties are often important for the recognition and binding of immune cells (Gu, 2007), it is possible that the glycosylation of NIF is important for its binding to adhesion molecules which contributes to its inhibition of leukocyte recruitment. Apart from contributing to biological functions, the correct glycosylation of proteins may also be required to induce an appropriate immune response, which is important for vaccination studies. Therefore, it is highly feasible to produce recombinant VALs which are glycosylated, in which case the utilisation of eukaryotic expression systems may be more appropriate.

Popular and established eukaryotic expression systems include yeast (e.g. *Pichia pastoris*) and insect cells (e.g. baculovirus with insect cell lines such as Sf21). Both types of cells are able to N-glycosylate proteins at the Asn-X-Ser/Thr motifs, in a manner that is usually similar to higher eukaryotes (Feldmann, 2007). Although both cell types are also able to perform O-linked-glycosylation (Invitrogen, 2010), the resulting glycosylation patterns could however be different to nematodes, as analysis of O-glycan structures in *C. elegans* has revealed unusual patterns (Guerardel *et al.*, 2001). Both yeast and insect cells usually produce high yields of proteins with the added advantage of being endotoxin (LPS)-free, thus making the products more



suitable for use in cellular immunology studies and vaccination. VAL variants of *Heligmosomoides polygyrus* have been expressed in insect cells by the Maizels lab, with excellent results (Murray *et al.*, 2010). Mammalian cell expression systems (e.g. Chinese hamster ovary, CHO cells) are also good for producing proteins which are often expressed in their native form, but is however associated with high cost and low yield (Verma *et al.*, 1998).

During my project I have additionally made multiple attempts to express the full length versions of *NbVAL3* and *NbVAL8* in *P. pastoris*. The *NbVAL3* and *NbVAL8* inserts were previously cloned into the vector pCR2.1-TOPO by Huang (2010). I then excised the inserts and ligated them into the yeast vector pPICZ $\alpha$ A, and verified that the sequences were in frame. I next attempted to transform the vectors into *P. pastoris* X-33 strain by electroporation and lithium chloride transformation. Although positive colonies were obtained, the transformants did not produce the target VAL proteins, although it was unclear why this was so.

The possibility of expressing parasitic nematode genes in *C. elegans* is a very attractive option. Since the basic biochemistry and genetics of *C. elegans* is similar to parasitic nematodes, it is highly likely that the recombinants produced would resemble the native structure and have the correct post-translational modifications. Kwa *et al.* (1995) showed that it is possible to stably express and maintain a  $\beta$ -tubulin gene, *tub-1* from *H. contortus* in *C. elegans* by microinjection of DNA into germ cells of the gonad. The expressed product was functional and able to rescue the *C. elegans* orthologous mutant phenotype, demonstrating that the protein was correctly folded and processed. In another study, a cystatin gene from *A. viteae* was also successfully expressed in *C. elegans* (Pillai *et al.*, 2005). Expression of a *H. contortus* cathepsin L protease, *Hc-cpl-1*, in *C. elegans* restored normal embryogenesis in mutants for the orthologous gene (Britton and Murray, 2002). The expressed protease was enzymatically active and glycosylated, and recombinant protein from transgenic *C. elegans* can be produced and purified in sufficient amounts for vaccination studies to be carried out (Murray *et al.*, 2007). These studies highlight the potential of using *C. elegans* as a surrogate system in producing parasitic nematode proteins for functional and biochemical analysis, as well as vaccine testing.

Keeping in mind that the VAL proteins are ultimately to be mass produced as vaccine targets, one must take a pragmatic view in terms of the cost, efficiency and safety of batch production. Bacterial systems are undesirable, because of the concern of LPS contamination, which can alter the physiology and cause adverse effects if injected into humans. Yeast systems such as *P. pastoris* are ideal, since they can give high yields at a relatively low cost, which is important as hookworm is an infection of the world's poorest. Insect and nematode systems may have a higher possibility in expressing products more similar to the native form, but at this stage they are still very expensive and much more difficult to deal with on a large scale, and therefore more suited to production of proteins for research purposes rather than for bulk generation of vaccine.

### ***VALs and protective immunity***

Through serum ELISA and lymphocyte proliferation experiments with multiply-infected mice, it was demonstrated that *NbVAL7* displayed significant immunogenicity, with both cellular and humoral responses activated at higher levels than *NbVAL4*. These responses seemed to be characteristic of Th2-biased immunity, with significantly elevated levels of antigen-specific serum IgE, IgG1 and IgA, although cytokine response was not detectable. Importantly, these experiments also showed that the immune components, primed by multiple infection, can respond to the recombinant *NbVAL7* produced in SHuffle cells. These lines of evidence support the rationale for using *NbVAL7* in vaccination experiments.

Although VAL proteins have been shown to protect against hookworm infection in a variety of systems, this effect was not demonstrated in my experiments with *NbVAL7*. There could be several reasons for this, the most obvious being that immune responses to *NbVAL7* are not protective for *N. brasiliensis* infection. Although a great body of evidence exists in the literature to support the protective effects of VAL antigens, there have been cases in which they did not elicit protection. Another possibility lies in the conformation of the recombinant protein used in the vaccination experiment, which has not been tested by NMR or X-ray crystallography. Although the *NbVAL7* proteins generated from SHuffle cells were soluble and stable from

expression to dialysis, this is not proof of correct conformation. Conformational epitopes are likely to be important for eliciting protection, as observed in several cases in which vaccination with VALs produced in *E. coli* failed to elicit protection. For example, vaccination with the baculovirus expressed Hc40 from *H. contortus* conferred protection in sheep, while its *E. coli*-expressed counterpart did not (Sharp and Wagland, 1998). Immunisation with *Bm*-VAL-1 produced in *E. coli* also did not protect jirds from a challenge infection with *B. malayi* (Murray *et al.*, 2001). On the other hand, vaccination with the baculovirus expressed *Oo*-ASP1 failed to elicit protection, even though vaccination with the native protein purified from *O. ostertagi* resulted in a 74% reduction in faecal egg counts (Geldhof *et al.*, 2008). Antisera to the recombinant protein (which was insoluble) failed to recognise its native version, suggesting that the baculovirus produced-protein was also incorrectly folded or lacked post-translational modifications essential for recognition.

In addition, only the N-terminal domain of the double-domain *Nb*VAL7 was cloned and expressed as a recombinant protein, and perhaps important epitopes for eliciting protection exist other parts of the protein, or the presence of the whole molecule may be required. Lastly, the recombinant proteins produced in *E. coli* were not glycosylated, which could be an important factor for immune recognition. Indeed, most of the successful vaccination trials have made use of VALs produced in yeast, including the studies involving ASP-2 in dogs and hamsters (Bethony *et al.*, 2005; Goud *et al.*, 2004). Moreover, antibodies produced during vaccination of sheep with *H. contortus* secreted proteins were found to recognise predominantly glycan moieties (Vervelde *et al.*, 2003). Interestingly, such glycan-specific antibody responses are most prominent for the isotypes IgG and IgA, but only moderate for IgE, perhaps suggesting that such epitopes may have the potential to generate a protective immune response without eliciting IgE-related hypersensitivity reactions. Furthermore, it was found that a quarter of the antibodies generated against the *H. contortus* gut antigen H11 target carbohydrate epitopes (Newton and Meeusen, 2003). Immunisation of sheep with native H11 purified from *H. contortus* induces a 90% reduction of worm burden during challenge, but little protection can be elicited by vaccination with recombinants produced in *E. coli* or baculovirus.

There is also room for consideration with regards to the vaccination procedures. The dosage for vaccination could be a variable, as could the choice of adjuvant. Alternative adjuvant candidates, such as the oil-water emulsion type ISA-720 (Seppic) and AS02 (GSK) could be further explored (Hotez *et al.*, 2003). More importantly, it should be noted that mice are not the natural host of *N. brasiliensis*. The decision to use mice rather than rats, their natural host, for vaccination is based on that fact that the immunological reagents to screen immune responses are far superior for mice in terms of variety and availability. Indeed, it has been possible to carry out a wide range of experiments to test the immune response of the mice pre- and post-vaccination with convincing data. Nevertheless, this decision may have compromised the quality of data involving parasite recovery after challenge, as the parasite life cycle in the mouse is truncated much earlier.

The original plans were to produce a mouse-adapted strain of *N. brasiliensis* for these experiments, a procedure claimed to be possible according to the literature (Kassai, 1982). This involves passaging parasites through the mouse and subsequent selection of the surviving progeny until a stable population of mouse-adapted worms are produced, which would normally require 4-6 passages. This procedure has been repeated 6 times in our laboratory over four months, and in only one infection have we been able to recover a very small number of L3, which were then lost again through re-infection. Isolation of adult worms from the gut of the infected mice revealed that a portion (about 20%) of the parasites were able to transit through the mouse, but the resulting adults were infertile and unable to produce eggs, explaining the extremely low recovery of L3. Therefore, in our experiments we were only able to assess the effect of vaccination on worm burden and viability, but not egg output. It also could be possible that the different physiology of the mouse to the rat may have affected the infection process, confounding the results in ways unknown. The vaccination experiments should be replicated in rats to assess vaccine efficacy in the natural host.

VAL proteins are secreted antigens, and one possible mode of vaccine action could be based on production of neutralising antibodies, inactivating VAL protein function. As discussed, the functions of VALs remain undefined. The fact that vaccines based on VALs have been effective in a number of models could be an indication that they do

have important functions. For functional studies with recombinant proteins to generate reliable and useful data, it is important for the expressed product to resemble the native protein as much as possible in structure and activity. An appropriate expression system should be chosen, and the conformation of expressed products verified by structural methods such as X-ray crystallography or NMR prior to experimentation.

The data showing that neutralising antibodies to ASP-2 were able to inhibit hookworm penetration forms part of the reasoning for choosing *Na*-ASP-2 to be the vaccine candidate by the Human Hookworm Vaccine Initiative (Bethony *et al.*, 2005; Goud *et al.*, 2005), with the implication that ASP-2 perhaps plays a role in skin penetration at the onset of infection. This basis for postulate seems to be the homology between the nematode VALs and Ves v 2, a venom allergen protein in yellow jacket wasps which is a hyaluronidase, an enzyme which has the capacity to break down mammalian skin structure (Henriksen *et al.*, 2001). It also fits the theory that VAL proteins play important roles in the transition to parasitism and onset of infection. However, these results were not replicated in my current study with *N. brasiliensis* using any of the antisera to *Nb*VALs. In addition, the hyperimmune serum, a concoction which include antibodies to most secreted proteins and other antigens of *N. brasiliensis*, also did not inhibit skin penetration. This suggests that immune protection is not effected by antibody-mediated inhibition of penetration of the skin. Indeed, in studies of *N. brasiliensis* reinfection of immune animals, the site of immune priming and elimination of the worms occurs almost entirely at the lungs (Harvie *et al.*, 2010). These findings suggest that design of vaccination strategies against strongylid nematodes target immune responses in the lungs rather than the skin.

### ***VALs and Hypersensitivity***

Production of IgE by B cells is an effector response common in parasitic infections, but can also sensitise mast cells for type I hypersensitivity reactions through its effectively irreversible binding to their FcεRI receptors. Cross-linking of bound IgE molecules on re-encounter of its antigen causes degranulation and release of

mediators from mast cells and/or basophils, resulting in allergic reactions, which can potentially be life-threatening in anaphylactic cases. For a vaccine candidate such as *NbVAL7* to have the capacity to induce production of IgE is potentially dangerous, because the recipient will have to undergo repeated exposure to the antigen through multiple vaccination boosts as well as possible future and/or previous encounter with the pathogen, stimulating clonal expansion of IgE every time.

It was demonstrated in this chapter that *NbVAL7*- and *NbVAL4*-specific IgE were produced in mice infected with *N. brasiliensis*. While this confirms the immunogenicity of *NbVALs*, it also highlights a potential drawback in their use as vaccines. Their capacity for inducing Type I hypersensitivity reactions was confirmed by the *in vivo* skin tests, in which immediate cutaneous anaphylaxis to *NbVAL7* and *NbVAL4* were clear in 4 out of 10 multiply-infected mice. These data mirror the results from the human vaccination trials with *Na-ASP-2*, in which previously infected human individuals exhibited Type I hypersensitivity reactions upon immunisation, an effect which correlated with increased IgE levels (Bethony *et al.*, 2008a).

It is additionally striking that immunisation with recombinant *NbVAL7* resulted in high levels of antigen-specific IgE in naive mice without any prior exposure to the parasite. This suggests that the allergenic properties of *NbVAL7* are likely to be associated with the antigen itself, rather than just being associated with the general type 2 response mounted by helminth infection. This resonates with the results from the first *Na-ASP-2* vaccination trials with healthy volunteers unexposed to hookworm, which reported statistically insignificant but consistent trends of IgE levels increasing with immunisation boosts and ‘mild to moderate injection site reactions’ of pain, swelling, erythema and pruritus (Bethony *et al.*, 2008b). Concerns associated with these findings were verified in later trials with individuals previously exposed to hookworm (Bethony *et al.*, 2008a).

On the other hand, there are many who support the view that IgE production is a double-edged sword which plays an integral part in helminth immunity. In vaccination experiments with VAL antigens from *H. contortus* (Hc24), *A. caninum* (Ac-ASP-1) and *Necator americanus* (Na-ASP-1), protection from parasite challenge

was highly correlated with IgE (Ghosh and Hotez, 1999; Hotez *et al.*, 2003; Kooyman *et al.*, 2000). Parasitological and serological surveys in hookworm endemic areas also pointed to a correlation between high *Na*-ASP-2-specific IgE levels and low hookworm intensity (Bethony *et al.*, 2005). It could be possible that IgE production is just an effect manifested by repeated exposure to the allergenic VAL antigens, or it may be the reason why VAL proteins are protective antigens. Although expulsion of GI nematodes are generally antibody-independent, IgE has been linked to the weakening of larval and adult worms, which may impair survival and fecundity, as well as mast cell responses (Gurish *et al.*, 2004; Pritchard *et al.*, 1995). In schistosomiasis, resistance to reinfection is correlated to high IgE levels, which also contributes to the killing of larval schistosomes (Hagan, 1993). Immunisation with schistosomula-specific monoclonal IgE resulted in elimination of the parasite through macrophage action, and IgE has also been shown to mediate eosinophil action in the killing of *S. mansoni* (Gounni *et al.*, 1994; Zhang and Mutapi, 2006). Although IgE action could possibly contribute to the protective effects of a vaccine, it may not be an absolute requirement, as immunisation with other antigens such as the *Ac*-MTP1, another L3 secreted protein, also resulted in protection but correlated with IgG2 rather than IgE levels (Hotez *et al.*, 2003).

It is perhaps not surprising that VAL proteins are allergenic, considering their homology to the vespid venom allergen proteins. True to their namesake, these proteins are the major allergy-inducing components present in the venom of a range of biting insects including bees, yellow jacket wasps, hornets, fire ants, mosquitoes, sandflies, tsetse flies, and even snakes (Cantacessi *et al.*, 2009). Structural studies have found conserved regions between these proteins which are suggested to constitute major B cell epitopes for an IgE response (Henriksen *et al.*, 2001). It is possible that these epitopes could also be conserved in the nematode proteins, causing hypersensitivity.

Collectively, these data suggest that the VAL antigens, with their tendency to induce type 1 hypersensitivity, are not safe for use in vaccination to protect against hookworm infection. The Human Hookworm Vaccine Initiative has halted vaccination trials for *Na*-ASP-2 and has moved on to testing new targets (Periago, 2010). However, Alex Loukas' group are currently analysing the allergenic epitopes

on VAL proteins, in the hopes that the implicated sites can be modified to downplay its allergenic properties without compromising its protective effects. If this could be achieved, then the VAL antigens, having produced promising results, could still have future use in vaccine formulations against helminth parasites.

### ***Chapter conclusions***

The immunogenicity and protective effects of vaccination with VALs has been demonstrated in several systems, although the latter has not been replicated in my studies with *NbVAL7* produced in *E. coli* SHuffle. It is clear that the expression of soluble, pure and structurally accurate VAL proteins is important, and key to producing reliable data on biological functions. Although research on VAL proteins as human hookworm vaccine candidates has reached a bottleneck for safety reasons, it is still important that the functions of VAL proteins be found. Considering the widespread distribution of VAL proteins in parasitic nematodes, elucidating their functions may not only improve our understanding of host-parasite interactions, but also inform of the design of future vaccination strategies.



# **Chapter 6**

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## **Conclusions**

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Secreted proteins form the primary interface between parasite and host, and they are important molecules for infection, involved in a range of functions during different stages of the parasite life cycle including host invasion, immunomodulation and survival. The analysis of secreted proteins of *N. brasiliensis* is an underlying theme to this project. The secretion profile of *N. brasiliensis* has been analysed by 2-DE for the first time, and showed that the proteins secreted by the larval and adult stages differs not only quantitatively but qualitatively. The composition of adult secreted proteins showed higher complexity, with a wide molecular weight range, whereas proteins secreted by L3A lie mostly between 15-37 kDa, although a large number of proteins were found within this region in relatively smaller quantities. The analysis of larval secreted proteins has been of higher interest to our laboratory than those of adults, as they are likely to contain factors relevant to parasite infectivity. Studying proteins secreted by larvae would thus improve our understanding of possible invasion factors and the early establishment of the host-parasite relationship, providing an opportunity to design intervention and vaccination strategies which can target worms at the onset of infection.

In the absence of a genome sequence, a global proteomic characterisation of all proteins in the mixture would be technically challenging. Besides, it is unlikely that all secreted proteins will have equal contribution to parasite infectivity. When the field-isolated J strain of *N. brasiliensis* was donated to our laboratory, initial life cycle passages revealed that it was noticeably more productive than our laboratory passaged W strain. We originally suspected that this was due to greater infectivity, and a comparison of larval secreted proteins of the two strains may highlight molecules which are important in infectivity. However, comparative analysis of infection dynamics showed that the two strains were equally infective, and the difference in productivity was attributable to the higher adult fecundity and persistence of the J strain. Thus factors affecting parasite fecundity and survival may instead be differentially manifested by the two strains at the adult stage. Although not an original aim of the project, this was an interesting phenomenon which I sought to further analyse. The host response to infection with the two strains was found to be similar in mode and magnitude, indicating that factors which regulate the differences in infection dynamics were likely to be intrinsic to the worms. This also suggests that the

two strains are unlikely to show overt differences in their capacity to modulate the host immune response. It is however possible that the two strains could show differential capacity to resist host expulsion mechanisms, and thus adult secreted proteins of the two strains were assayed for acetylcholinesterase and nucleotide metabolising enzyme activities, but the results did not explain the differences in infection dynamics. Although some differences were found between the secretion profiles of the two strains resolved by 2-DE, we decided that a proteomics project to qualify such differences may not be particularly informative. Moreover, since the genome sequence of *N. brasiliensis* was not available, identification of proteins by peptide mass fingerprinting (PMF), a technique which compares the mass of enzyme-cleaved peptide fragments with theoretical peptide masses predicted in databases, was an impractical strategy. Thus there would be a need to obtain the actual amino acid sequences of the proteins of interest by tandem mass spectrometry and N-terminal sequencing, which is a lot more time consuming, and even then the elucidation of protein identity was not guaranteed due to the lack of genomic and proteomic data.

Studies on the infection dynamics of the two strains did however provide interesting insights as to how laboratory passage may affect the characteristics of a parasite strain. Since parasite fecundity and most likely survivorship are compromised after repeated high-dose passage, data generated from such laboratory strains may misrepresent natural infection and bias epidemiological models. The W strain has therefore been replaced with the J strain for parasite work on *N. brasiliensis* exclusively in our laboratory, as it probably more closely represents natural infection.

Activation is an important event central to the infective process which is associated with the transition of a parasitic nematode from its free-living phase to its parasitic phase. This process depends sensitively on the parasite's ability to sense and respond to host cues appropriately in order to maximise its chances of infection and avoid mis-activation in the wrong context. I have shown that both thermal and chemical input can be important for activation. Notably, a temperature cue at 37°C was sufficient to activate larval feeding to over 90% and initiate the synthesis and secretion of proteins in *N. brasiliensis*. Although chemical host cues such as rat serum and rat skin did not synergise with the temperature cue to enhance these activities, in chemotactic assays *N. brasiliensis* L3 was found to be attracted to an aqueous fraction of rat skin, which

also induced the immediate release of a bolus of pre-synthesised protein, independent of temperature. This may suggest that thermal and chemical cues can be processed independently of each other, leading to different events during activation. Furthermore, such protein release is likely to be important for parasite invasion, like the hydrolytic enzymes involved in skin penetration (Hotez *et al.*, 1990; Salter *et al.*, 2000; Williamson *et al.*, 2006). However, recent evidence has shown that the protective responses which lead to immune attrition of *N. brasiliensis* occurs primarily at the lungs (Harvie *et al.*, 2010), suggesting that vaccination strategies for hookworms may be better to focus instead on stimulating protective immune responses at this site.

Proteins secreted by activated larvae were found to be highly immunogenic during infection, indicating that they are recognised by the host immune system and may possibly contribute to host immunity. It is particularly noteworthy that out of approximately 90 protein spots resolved by 2-DE, only 8 of them reacted with immune serum from infected rats. Moreover, the strength of reactivity was not correlated with the quantity of protein. This differential recognition suggests that such proteins may have particular significance in immunity to nematodes, which would be interesting for further studies to test if they may be protective antigens. Immunoblotting has been the method of choice in sampling for immunogenicity, and has been successful in elucidating a number of antigens protective to parasitic nematodes (Knox, 2000). However, serum antibody reactivity may not always be indicative of an ability to induce protective responses, especially since antibody responses generally do not play a large part in nematode immunity (Else and Grecnis, 1996). Alternative methods to identify antigens associated with protective immunity may focus on cellular reactivity, such as T cell Westerns (Haig *et al.*, 1989; Knox, 2000), or use antibody probes collected at the local sites of infection such as the mucosal surface or the draining lymph nodes.

The effect of RELMs on chemotaxis and feeding in *N. brasiliensis* was investigated, since there has been evidence which suggested that such activities were inhibited by RELM $\beta$  in parasitic nematodes (Artis *et al.*, 2004; Herbert *et al.*, 2009). In particular, it was interesting that RELM $\beta$ , a molecule whose cellular distribution suggest that it could only affect adult worms of the intestinal stage (Steppan *et al.*, 2001), has been

reported to inhibit the chemotactic responses of larvae. In my experiments such inhibition of chemotaxis was not demonstrated in *N. brasiliensis* L3, nor was feeding in either L3 or adults. Considering that a host deficiency in RELM $\beta$  result in a decreased ability to clear adult worms from the host intestines (Herbert *et al.*, 2009), its specific role in nematode immunity should be further examined.

The discovery of VAL proteins in *N. brasiliensis* provided an opportunity to test the efficacy of a secreted protein as a vaccination candidate. It was an initial aim to use a protein secreted at the larval stage for vaccination, and *NbVAL7* was chosen since it was identified in L3A-secreted proteins through Western blot analysis. Although it was found that *NbVAL7* was immunogenic during infection, immunisation with the recombinant protein produced in *E. coli* SHuffle cells did not protect mice from a challenge infection. These results were however complicated by the question of whether the recombinant proteins were correctly folded. Due to their high cysteine content, it has been noted by several laboratory groups that correct protein folding is a particular challenge for this family of proteins (Hotez *et al.*, 2003). The role of glycosylation on the function and protective properties of VALs are also unclear. Thus a focus on the production of recombinants which are comparable to the native protein in structure and post-translation modifications should be the priority before further experimentation on VAL proteins. Achieving this, the interpretation of functional and immunological data can be carried out with less ambiguity. Considering that the eight variants of *NbVALs* show differences in stage-specific expression, it would be interesting if their functions could be compared.

The discovery that *NbVAL7* and *NbVAL4* elicit antigen-specific IgE production and Type 1 hypersensitivity indicates that these proteins may not be suitable as vaccine candidates due to safety reasons. The demonstration that VAL-specific IgE can be induced without exposure to the parasite also suggests that the allergenic properties lie within the molecule itself. These results raise doubts about the suitability of VALs as vaccine components caused by the hypersensitivity reactions observed during the vaccination trials in humans (Bethony *et al.*, 2008a; Bethony *et al.*, 2008b). I have also shown that IgE specific to adult and larval secreted proteins were prominently produced during infection with *N. brasiliensis*, and this additionally highlight the

potential safety concerns which should be kept in mind when selecting nematode secreted proteins as candidates for vaccination.

In conclusion, this project has taken a broad approach in answering specific questions regarding secreted proteins in *N. brasiliensis*, with relevance to parasite infectivity and immunity. This project also improved our understanding of nematode secretions, particularly at the larval stage, as research efforts from other groups which study nematode secreted proteins have mostly focused on the adult stage. More detailed functional studies of larval secreted proteins will not only assist in development of anti-nematode vaccines, but should also yield valuable information on the processes involved in invasion and infection of the mammalian host.

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