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Secreted proteomes of different developmental stages of the gastrointestinal nematode *Nippostrongylus brasiliensis*

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Running title: Secretome of *Nippostrongylus brasiliensis*

Abbreviations

BLAST, Basic Local Alignment Search Tool

CAP, Cysteine-rich protein, Antigen 5, Pathogenesis-related protein 1 domain

DTE, Dithioerythritol

emPAI, exponential modified protein abundance index

ESP, Excretory/secretory products

GO, Gene Ontology

IAM, Iodoacetamide

NEx, whole worm extract

OGE, OFF-GEL electrophoresis

SCP, Sperm coat protein

TAPS, Tpx-1, Antigen 5, Pathogenesis-related protein 1, SCP-like

VAL, Venome allergen/*Ancylostoma* secreted protein-Like

Summary

Hookworms infect more than 700 million people worldwide and cause more morbidity than most other human parasitic infections. *Nippostrongylus brasiliensis* (the rat hookworm) has been used as an experimental model for human hookworm because of their similar life cycles and ease of maintenance in laboratory rodents. Adult *N. brasiliensis*, like the human hookworm, live in the intestine of the host and release excretory/secretory products (ESP), which represent the major host-parasite interface. We performed a comparative proteomic analysis of infective larval (L3) and adult worm stages of *N. brasiliensis* to gain insights into the molecular bases of host-parasite relationships and determine whether *N. brasiliensis* could indeed serve as an appropriate model for studying human hookworm infections. Proteomic data were matched to a transcriptomic database assembled from 245,874,892 Illumina reads from different developmental stages (eggs, L3, L4 and adult) of *N. brasiliensis* yielding ~18,426 unigenes with 39,063 possible isoform transcripts. From this analysis, 313 proteins were identified from ESPs by LC-MS/MS - 52 in the L3 and 261 in the adult worm. Most of the proteins identified in the study were stage-specific (only 13 proteins were shared by both stages); in particular two families of proteins – astacin metalloproteases and CAP-domain containing SCP/TAPS - were highly represented in both L3 and adult ESP. These protein families are present in most nematode groups, and where studied, appear to play roles in larval migration and evasion of the host's immune response. Phylogenetic analyses of defined protein families and global gene similarity analyses showed that *N. brasiliensis* is more closely related to human hookworm than are other model nematodes including the murine gastrointestinal parasite *Heligmosomoides polygyrus*. These findings validate

Secretome of *Nippostrongylus brasiliensis*

the use of *N. brasiliensis* as a suitable parasite for the study of human hookworm infections in a tractable animal model.

Introduction

Nematodes belonging to the suborder Strongylida are, from an epidemiological and a socio-economic perspective, amongst the most relevant parasites in the world. Within this suborder, species from the genera *Necator* and *Ancylostoma* (also known as hookworms) infect more than 700 million people in tropical areas, and are considered to cause one of the most important human helminth infections along with schistosomiasis in terms of disability-adjusted life year's loss (1-3).

Nippostrongylus brasiliensis (order Strongylida, superfamily Trichostrongyloidea) is a soil-transmitted nematode, also known as the 'rodent hookworm' because of the similarities in life cycle and morphology between this species and the human hookworms *Necator americanus* and *Ancylostoma duodenale*. For these reasons, *N. brasiliensis* has been extensively used as a model to study the immunobiology of gastrointestinal nematode infections (4-6). Like the human hookworms, the life cycle of *N. brasiliensis* is direct with no intermediate hosts; first-stage rhabditiform larvae (L1) hatch from eggs after 24 hours at optimal conditions, and develop through two moults to become the infective stage, the filariform L3. L3 penetrate the skin of the host and migrate through the subcutaneous connective tissue where they enter the circulatory system and travel to the lungs before exiting into the alveolar spaces and moulting to the L4 stage. From here they migrate up the trachea and are swallowed, finally entering the gastrointestinal tract as L4 larvae and maturing to sexually dioecious male and female adults in the small intestine where they begin to feed and mate.

The *N. brasiliensis*-rodent model has been widely used as a model for human hookworm disease (4, 7-9). As a consequence of L3 migration through the tissues, *N. brasiliensis* stimulates a profound T helper type 2 (Th2) immune response in the skin, lungs and intestine, including IgE production increased mucus production and inflammatory cell infiltrates consisting of eosinophils, mast cells, basophils and innate lymphocytes (10-16). Despite an abundance of studies addressing the mechanistic aspects of rodent immunity to *N. brasiliensis* infections, there is a distinct paucity of molecular information about the parasite itself. A search of the NCBI database for *N. brasiliensis* retrieves only 116 proteins (most of them redundant), and an early transcriptomic analysis (pre-Next Generation Sequencing technologies) described ~1300 expressed sequence tags corresponding to 742 distinct genes (17).

Herein we present the first high-throughput proteomic characterization of the proteins present in the excretory/secretory products (ESP) of *N. brasiliensis* infective stage L3 and intestinal-dwelling adult worms based on a full exploration of the transcriptome using Illumina-based sequencing technology. Large-scale data comparisons between the secreted proteome from *N. brasiliensis* and available genomic and proteomic data for *N. americanus* were performed (18). This comprehensive analysis of the proteins and mRNAs produced by *N. brasiliensis* provides new insights into the molecular interactions at the host-parasite interface and highlight the molecular similarities between *N. brasiliensis* and *N. americanus*, emphasising the utility of this model rodent nematode for exploring the immunobiology of hookworm infections, and as a model for the discovery and development of new therapeutic approaches to controlling gastrointestinal nematodes.

Experimental procedures

Parasite material

N. brasiliensis was maintained in Sprague-Dawley rats as previously described (4, 19) and in accordance with UK Home Office and local Ethical Review Committee approvals. Infective L3s were prepared from two-week rat faecal cultures with careful preparation to ensure 100% viability. Adult worms were recovered from gastrointestinal tissue using a Baermann apparatus on day 6 post-infection following subcutaneous injection of 3,000 infective L3. In addition, eggs and lung-stage larvae were included in the transcriptomic analysis to ensure that transcripts encoding proteins present in the subsequent L3 and adult worm secretomes were fully represented.

RNA sequencing and transcript annotation

The RNA extraction was performed as described previously by Marcus et al. (17). Briefly, total RNA was extracted from different stages of *N. brasiliensis* (egg, L3, L4 and adult) and homogenized in 1 ml Trizol (Gibco Life Technologies). The homogenate was centrifuged (12,000g, 10 min), and the supernatant extracted with chloroform before isopropanol precipitation of RNA from the aqueous phase and DNase treatment. Polyadenylated (PolyA+) RNA was purified from 10 mg of total RNA, fragmented to a length of 100-500 bases, reverse-transcribed to cDNA, adaptor-ligated and paired-end sequenced on a Genome Analyzer II (Illumina), obtaining paired-end reads with a length of 108 bp each. The resulting data passed a quality control (QC) where sequencing adapters and reads with low quality (< 30 PHRED) were removed. Transcript reconstruction was performed using the Trinity assembler (rev 2013-02-25) (20) using default parameters, from a pool of reads for all life

stages. Also, downstream analysis for transcript abundance estimation and differential expression between life stages was performed following the protocol described in (21). Reconstructed transcripts were annotated using the Trinotate pipeline (<http://trinotate.sourceforge.net/>), a blastx search against the NEMBASE4 (<http://www.nematodes.org/nembase4/>), and against the entire genome sequence of the human hookworm *N. americanus* (18) to confirm their origin and rule out contamination from host or bacterial sources. Proteins were then conceptually translated from the predicted coding domains of individual cDNA sequences.

Isolation of ESP

Adult worms and L3 were recovered in saline at 37°C, washed 6× in saline and 6× in RPMI-1640 containing 100 µg/mL penicillin and 100 U/mL streptomycin (complete medium), and viability was determined by microscopy. The collection of ESP from L3 larvae and adult worms *in vitro* followed previously described protocols (6, 22). Briefly, parasites recovered as above were cultured in complete medium supplemented with glucose (to 1%), at 37°C in an atmosphere of 5% CO₂. Supernatants were collected daily from worms cultured for 2-7 days, pooled, centrifuged at 400×g for 5 min to remove any eggs, passed through a 0.2 µm Millex filter, concentrated over a 10,000 Da Amicon membrane, and stored at ~1.0 mg/mL in PBS at -80°C until required. *N. brasiliensis* adult worm somatic extract (NEx) was prepared by homogenisation on ice of freshly-isolated adult worms in PBS followed by centrifugation at 12,000×g for 30 min and recovery of the supernatant.

2D electrophoresis and in-gel digestion

The ESP of *N. brasiliensis* was subjected to two-dimensional gel electrophoresis (2DE) and silver stained as described previously (23). Briefly, 10 μ g of protein was resuspended in 125 μ L of rehydration buffer (7M Urea (Electran), 2M thiourea (BDH), 4% CHAPS (Sigma, St. Louis, USA), 65mM DTE (Sigma), 0.8% IPG buffer 3-10 (GE Healthcare,) and trace bromophenol blue (Sigma)), and used to rehydrate a 7 cm strip pH 3-10 (Immobiline; GE Healthcare) for 14 h at 20°C. Proteins were then subjected to isoelectric focusing on an IPGphor (Pharmacia Biotech,) at 20°C using the following program: (i) 500 V for 30 min; (ii) 1000 V for 30 min; (iii) gradient to 8000 V for 6 h; (iv) total ~20 kVh. Strips were reduced and alkylated as described previously (24), and proteins were separated by molecular weight using NuPAGE 4-12% Bis-Tris ZOOM gels (Invitrogen) and Nu-PAGE MES SDS running buffer (Invitrogen) for 2 h 10 min at constant 100 V. Gels were silver stained using PlusOne (Amersham Biosciences) with a few modifications as described by Yan et al. (25).

In-gel digestion of the 56 spots sliced from the gel was performed as described by Mulvenna et al. (26) with some modifications. Gel spots were washed in 50% acetonitrile, 25 mM NH₄CO₃ for 5 min three times at 37°C and then dried under a vacuum centrifuge. The spots were then reduced in 20 mM dithiothreitol (DTT) for 1 h at 65°C, the supernatant was removed and samples were alkylated by the addition of 55 mM iodoacetamide (IAM) and incubated at room temperature in darkness for 40 min. Gel spots were washed 3 \times in 25 mM NH₄CO₃ and dried in a vacuum centrifuge. The dried spots were rehydrated with 20 μ L of 40 mM NH₄CO₃ containing 20 μ g/mL trypsin (Sigma) for 45 min at 22°C. An additional 50 μ L of 40 mM NH₄CO₃, 9% acetonitrile was added to the samples and incubated overnight at 37°C. The digest supernatant was removed from the spots, and residual peptides were removed from the gel slices by washing 3 \times 0.1% TFA for 45 min at 37°C. The original supernatant

Secretome of *Nippostrongylus brasiliensis*

and extracts were combined and dried in a vacuum centrifuge. Samples were desalted and concentrated using Zip-Tip® (Merck Millipore) and eluted in ~5 µL of 50% acetonitrile 0.1% TFA before mass spectral analysis.

Two-dimensional differential in-gel electrophoresis

The adult and L3 ESP from *N. brasiliensis* (5 µg) were labelled with CyDye DIGE Fluor (minimal dyes) Cy3 (green) and Cy5 (red) respectively according to the manufacturer's instructions. Samples were combined and subjected to IEF using IPG strips (7 cm, pH 3-10 - source) under the following settings: rehydration for 14 h at room temperature, 300 V for 30 min, 1000 V for 30 min and gradient to 5000 V for 2 h and followed by a second dimension separation in a NuPAGE Novex 4-12% Bis-Tris ZOOM protein gel. The gels were scanned using Fujifilm Image reader FLA-5000 series V1.0.

OFFGEL electrophoresis

For peptide separation, OFFGEL fractionation was performed as described by Cantacessi et al. (27) with some modifications. A total of 100 µg of ESP and NEx products were resuspended in 2% SDS, 20 mM DTT and were incubated at 65°C for 1 h. Alkylation was then achieved by adding IAM to 55 mM and incubating the solution for 40 min in darkness at 22°C. The sample was co-precipitated with 1 µg of trypsin (Sigma) and the addition of 9 volumes of methanol and was incubated overnight at -20°C. The sample was centrifuged and the pellet was resuspended in 100 µL of 50 mM NH₄CO₃ and incubated for 2 h at 37°C. After the addition of an extra 1 µg of trypsin, the sample was incubated overnight at 37°C. The samples were then fractionated using a 3100 OFFGEL fractionator and OFFGEL kit (pH 3–10; 24-

well format) (Agilent Technologies) according to the manufacturer's protocols. The digested proteins were diluted in peptide-focusing buffer to a final volume of 3.6 mL and 150 μ L of sample were loaded into each of the 24 wells. The sample was focused in a current of 50 μ A until 50 kilovolt hours (kVh) was reached. Peptide fractions were collected, dried under a vacuum centrifuge and resuspended in 10 μ L of 0.1% TFA. Finally, samples were desalted using Zip-Tip® (Merck Millipore) and dried again under a vacuum centrifuge.

Mass spectrometry and protein identification

An AB SCIEX TOF/TOF 5800 mass spectrometer (Applied Biosystems) was used for acquiring MALDI-MS/MS data from gel spots, and samples without a positive identification were analysed by LC-MS/MS. ESP separated by OFFGEL were analyzed by LC-MS/MS on a Shimadzu Prominence Nano HPLC coupled to an AB SCIEX Triple ToF 5600 mass spectrometer (Applied Biosystems) equipped with a nano electrospray ion source. Six microliters of sample was injected onto a 50 mm 300 μ m C18 trap column (Agilent Technologies). The samples were desalted on the trap column for 5 min using 0.1% formic acid (aq) at 30 μ L/min. Peptides were then eluted onto an analytical nano HPLC column (150 mm x 75 μ m 300SBC18, 3.5 μ m, Agilent Technologies) at a flow rate of 300 nL/min and separated using a 35 min gradient of 1-40% buffer B followed by a steeper gradient from 40%-80% buffer B in 5 min. Buffer B contained 90/10 acetonitrile/ 0.1% formic acid, and buffer A consisted of 0.1% formic acid (aq). The mass spectrometer acquired 500 ms full scan TOF-MS data followed by 50ms full scan product ion data in an Information Dependent Acquisition, IDA, mode. Full scan TOFMS data was acquired over the mass range 350-1400, and for product ion ms/ms 80-1400 m/z ions observed in the

TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, ms/ms spectra of the resultant 20 most intense ions. The data was acquired and processed using Analyst TF 1.6.1 software (ABSCIEX, Canada).

Bioinformatic analysis of proteomic sequence data

Database searches were performed against NCBI nr database (March 2013 version) to detect possible contamination and on the peptide sequences predicted from the *N. brasiliensis* transcriptomic data (20,136 entries) using MASCOT search engine v4.0 (Matrix- Science). The parameters used for MALDI-TOF/TOF were: enzyme; trypsin; precursor ion mass tolerance = ± 0.8 Da; fixed modifications = methionine oxidation; variable modifications = carbamidomethylation; number of missed cleavages allowed = 2; charges states = +1. The parameters used for LC-TOF/TOF were similar except for the precursor ion mass tolerance = ± 0.1 Da and for the charges states = +2, +3. The results from the Mascot searches were validated using the program Scaffold v.4.2.1 (Proteome Software Inc., USA) (28). Peptides and proteins were identified using the Peptide Prophet algorithm (29), using a probability cut-off of 95% (peptides) or 99% probability (proteins), and contained at least two identified peptides (proteins) (30). Proteins containing similar peptides that could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony. A false discovery rate of <0.1% was calculated using protein identifications validated using the Scaffold program (v.4.2.1).

Proteins were classified according to GO categories using the program Blast2Go (31) and Pfam using HMMER v3.1b1 (32) and CD-domain, putative signal peptides and transmembrane domain(s) were predicted using the programs CD-Search tool (33),

SignalP (34) and TMHMM (35), respectively. Putative mannose 6-phosphate glycosylation sites were identified using the NetNGlyc server (36).

The similarity analysis was based on the model described by Parkinson and Blaxter (37) using an in-house built script as previously described (38) with the *N. americanus* predicted proteome (18), the secreted proteome of *Heligmosomoides polygyrus* adult worms (39) and the secreted proteome of *Brugia malayi* adult worms (40).

Phylogenetic analyses

Homologues of Sperm-coating protein (SCP)-like extracellular proteins (SCP/TAPS) and astacins, respectively, were identified from sequence datasets available for species of parasitic nematodes from Clade I-V (41) in the NCBI database (August 2013 version), as well as in the *N. brasiliensis* L3 and adult secretomes, the *N. americanus* predicted proteome (18) and the *H. polygyrus* adult secretome (39). The signature conserved protein motifs for SCP/TAPS (PF01400) and astacins (PF00188), respectively, were aligned using the MUSCLE software (Edgar, 2004). Best-fit evolutionary models for maximum-likelihood (ML) phylogenetic analyses of SCP superfamily and astacin-peptidase family amino acid sequences were predicted using ProtTest (42). The best-fit model inferred from the Akaike Information Criteria (AIC) was used in the amino acid dataset analyses. For each amino acid sequence alignment, ML and Bayesian Inference (BI) trees were derived using MEGA v.5.2 (43) and MrBayes 3.2.2 on XSEDE (44), respectively. The different trees were rooted using the RTVP-1 protein from *Homo sapiens* (GenBank accession number X91911) and the human astacin-like metallo-endopeptidase (GenBank accession number AAI07128) for the SCP/TAPS and astacin trees, respectively. The ML phylogenetic

trees of amino acid sequences for astacins and SCP/TAPS were constructed using the WAG model assuming uniform rates among sites (+G + I; i.e. including gamma, proportion of invariant sites, and +G respectively). For each ML analysis, the bootstrapped confidence interval was based on 1000 replicates. BI analyses alignments were run over 3,000,000 generations ('ngen = 3,000,000') with two runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains ('nchains = 4') and every 100th tree being saved ('samplefreq = 6000'). The parameters used were as follows: 'nst = 6', 'rates = invgamma', with MCMC left at default settings, 'ratepr = fixed' and 'burnin = 5000'. Consensus trees were constructed, with 'contype = allcompat' nodal support being determined using consensus posterior probabilities. All trees were displayed using FigTree v1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Transcriptomic database

A transcriptomic database was constructed from 245,874,892 Illumina reads derived from 4 developmental stages of *N. brasiliensis*. The initial assembly was filtered using read abundance criteria, where transcripts with at least 10 reads supporting the reconstruction, in at least one of the four developmental stages, were retained. After filtering, a total of 18,426 unigenes producing 39,063 possible isoform transcripts were obtained. These transcripts were used for conceptual translation, resulting in 48,583 ORFs that were annotated. A more detailed analysis of annotation, GO, Pfam EGGNOG and SignalP analysis will be presented in a future publication, however we noted that a total of 2,025 (10%) proteins from the predicted proteome of *N.*

brasiliensis presenting homologies with other nematodes were predicted to have a

signal peptide, 10.17% of which were inferred to be astacins and 10.96% were predicted to contain a SCP/TAPS domain (n = 222).

2DE of adult N. brasiliensis ESP

As an initial analysis of the secretome of *N. brasiliensis*, adult ESPs were electrofocused using 3-10 linear immobilized pH gradient strips and electrophoresed in NuPAGE 4-12% gels. After silver staining the gel, more than 100 spots could be differentiated, most of them located at a range of 8-216 kDa and a pH range of 4.4-10 (Fig. 1). Fifty-six spots detected in the 2D gel were analysed by MALDI-TOF/TOF and LC-MS/MS, one of which was discarded as human keratin. Subsequently, the search was performed using the proteome derived from transcriptomic analysis and only two of the spots (spots 10 and 34) were unable to be identified as their individual ions scores were below 38 ($p < 0.05$). The result of the mass spectrometry analysis is shown in Table 1, and a more detailed analysis, including the Pfam analysis, sequence information and annotation with NEMBASE and *N. americanus* genome is provided in Supplemental Table SI.

The most abundant family of proteins was a subfamily of the CAP-domain Cysteine-rich SCP/TAPS family (PF00188), otherwise termed Venom Allergen/*Ancylostoma* secreted protein-like (VAL) products (45, 46); no fewer than 37 of the 53 spots identified belonged to this family (namely spots 1-9, 11-21, 24-28, 31, 38-39, 41-49) and the corresponding protein sequences contained either a single or double CAP domain. The second most highly represented protein family was the globin-like superfamily (PF00042.17), with three spots containing a globin domain (e.g. spots 50, 51 and 53). Other proteins belonging to different families such as peptidases,

esterases, kinases or metabolic enzymes were identified and only two spots contained a domain of unknown function (spots 32 and 55). A total of 27 spots contained proteins for which a signal peptide could be identified in the transcriptome assembly, although not all N-terminal amino acid sequences were available for inspection; in addition 3 spots included proteins with at least one transmembrane domain (Table 1). Following NetNGlyc analysis, proteins from 36 spots had at least one putative mannose 6-phosphate glycosylation site.

Proteomic analysis of ESP

For a more comprehensive analysis of the secretome, ESP from L3 and adult worms, as well as and adult NEx were fractionated using OGE, and the tryptic peptides were analysed by LC-MS/MS, generating a total of 57,079 and 54,529 spectra for L3 and adults, respectively and 32,878 for NEx. Mascot searches were performed against the predicted proteins from the generated transcriptome, and Scaffold software (v.4.2.1) was used to validate the protein identifications. Using Scaffold, 52 proteins for L3 ESP and 261 and 275 for adult ESP and NEx were identified, respectively, by at least two peptides at a 99.0% probability and an estimated false discovery rate <0.1% (Supplemental Tables SII-SVII).

The number of shared proteins common to the different developmental stages is shown in Fig. 2a. In addition, a 2D-DIGE comparison of the *N. brasiliensis* L3 and adult ESP was performed in order to highlight the limited number of shared proteins common to both samples. The results (Fig. 2b) confirm the lack of common proteins in the ESP of these two developmental stages as very few spots overlapped in the gel. A total of 13 proteins were found in both L3 and adult ESP, whereas 102 proteins

were shared between adult ESP and adult NEx (Fig. 2a). Only 6 proteins were found in both L3 ESP and adult NEx. The complete lists of proteins and peptides identified for L3 ESP, adult ESP and NEx can be found in Supplemental tables SII-SVII, respectively.

The proteins identified from L3 and adult stages were annotated using Blast2GO (31). In total, 1,244 GO terms were returned from the 3 categories of the GO database: biological process, molecular function and cellular component (Supplemental Fig. 1). There were 88 and 768 biological process returned terms for L3 and adult ESP fractions respectively, and 'metabolic process' and 'multicellular organismal process' represented the most frequently predicted category in L3 (19.3% of the total) and adult ESP fractions (12.5%), respectively. Twenty-five (51%) molecular function terms were returned with 'catalytic activity' and 97 (43.3%) returned terms with 'binding', both representing the highest terms for L3 and adult ESP respectively. A total of 25 and 252 location terms were returned for the L3 and adult ESP secretomes, with the 'cell' location representing the most frequently predicted category in both fractions (44% and 37.6%, respectively) (Supplemental Fig. 1). The GO analysis of the ESP returned 30 and 50 molecular function terms for L3 and adult sequences, respectively (Supplemental Fig. 1). The most prominent terms in the L3 ESP were peptidase activity, protein binding and hydrolase activity. Similarly, protein binding, ATP binding and hydrolase activity were also key terms in adult ESP, whereas terms related to binding activities including heme binding, iron ion binding and oxygen binding were only prominent in the ESP of adult (Fig. 3).

The L3 and adult ESP proteome fractions were subjected to a Pfam analysis using HMMER v3.1b1 (32) and compared to the corresponding analysis of the transcripts from each stage that contained a signal peptide (Fig. 4). The greatest proportion (8 proteins) of the proteins from L3 belonged to the astacin peptidase family M12A (PF01400) (Fig. 4a), followed by a group of 5 proteins containing CAP domains (PF00188). In the adult ESP fraction, members of the SCP/TAPS family were the most highly represented (45 proteins), followed by astacin family M12A metallopeptidases (PF01400, 10 proteins) (Fig. 4b). The Pfam analysis of the L3 and adult predicted proteins from *N. brasiliensis* returned five major protein groups common to both samples, with protein kinase domain-containing proteins and tyrosine kinase proteins as the most highly represented (Fig. 4c, d). In contrast, the Pfam analysis of the predicted proteins containing a signal peptide showed a high representation of proteins with immunoglobulin domains, neurotransmitter domains and metallopeptidases in both L3 and adult (Fig. 4e, f). In addition, proteins with astacins and SCP/TAPS domains were abundant in the predicted secretome (Fig. 4e, f).

A quantitative measure of protein abundance is the exponential modified protein abundance index (emPAI), from which the 25 highest-scoring L3 ESP, adult ESP and adult NEx proteins are shown in Fig. 5 A-C respectively. Supplemental Tables SII, SIV and SVI show the complete list of proteins ranked by the emPAI. The most abundant constituent of the L3 ESP fraction is a metalloproteinase inhibitor (Merops family: I35), which is a tissue inhibitor of metalloproteases (TIMP), although some proteases and peptidases were also abundant in this fraction (Fig 5a). In addition, lysozyme-like proteins (GH family 25 lysozyme 2) were notable in L3 ESP, and also

detected in the adult ESP. In the adult ESP, the most abundant proteins were two proteins belonging to the SCP/TAPS family (m.233248 *Ancylostoma* secreted protein (ASP) and m.68479 ASP) and a transthyretin-like protein (m.436569 transthyretin-like protein 15). Other SCP/TAPS proteins were represented in adult ESP but were less abundant (Fig 5b). For the adult NEx proteome, the highest emPAI-scoring proteins were tropomyosin (m.198775) and actin (m.242612), although other globin-, structural-related proteins and metabolic enzymes were also highly represented (Fig. 5c).

Phylogenetic analysis

The Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of astacin and SCP/TAPS amino-acid sequence alignments resulted in consensus trees with clades supported by relatively high bootstrap values (ML) and posterior probabilities (pp; BI), respectively (Figs 6 and 7). For the astacins, the consensus tree consisted of two distinct monophyletic clades, with high bootstrap/pp support (i.e. 100/1.0), both including sequences from *N. brasiliensis* and *N. americanus* (Fig. 6). Within one of these clades, sequences from the rat and human hookworms formed paraphyletic clades, with one *N. brasiliensis* sequence (m72921) grouping to the exclusion of homologues from the free-living nematodes *Caenorhabditis briggsae* and *Caenorhabditis brenneri* (Fig. 6). The larger astacin monophyletic clade included sequences from all the remaining taxa included in the analyses, including representatives of the Spirurida (*Brugia malayi*), Ascaridida (*Ascaris suum*), Rhabditida (*Caenorhabditis elegans*, *C. briggsae* and *Strongyloides stercoralis*) and Trichocephalida (*Trichinella spiralis*) (Fig. 6).

For the SCP/TAPS, the ML/BI consensus tree included one main clade comprising all sequences analysed to the exclusion of a *T. spiralis* homologue (Fig. 7). While sequences from free-living nematodes (i.e. *C. elegans* and *C. remanei*), spirurids (i.e. *B. malayi*) and ascaridids (i.e. *A. suum*) clustered to the exclusion of most SCP/TAPS from strongylids, a single sub-clade included a conserved subset of SCP/TAPS from *N. americanus* and *N. brasiliensis* (Fig. 7); the latter clustered to the exclusion of other hookworm SCP/TAPS, as well as homologues from *H. polygyrus* (Fig. 7).

Comparative analysis of Nematoda ESP

To assess the similarity between *N. brasiliensis* and *N. americanus* ESP, a comparative analysis of the sequences based on the methodology described by Parkinson and Blaxter (37), but employing an in-house built script, was performed. A morphologically similar gastrointestinal nematode of rodents (*H. polygyrus*) and a non-related nematode (*B. malayi*) belonging to clades V and III, respectively (41) were included in order to determine the similarities between different nematodes and *N. americanus*. The results obtained are shown in Fig. 8. A total of 216 proteins were uniquely homologous to *N. brasiliensis* ESP, whereas 100 and 27 ESP had unique homology to *H. polygyrus* and *B. malayi* respectively. Furthermore, 70 proteins had homology with *N. brasiliensis* and *H. polygyrus* proteins, and only one and 2 proteins were homologous with both *N. brasiliensis* and *B. malayi* and with *H. polygyrus* and *B. malayi*, respectively. Interestingly, no proteins from *N. americanus* matched a common homologue in all three nematodes.

Discussion

In the present study, we provide the first proteomic analysis of the two key developmental stages of the rodent hookworm *N. brasiliensis*, coupled with a high-density transcriptomic profile of this parasite. *N. brasiliensis* is characterised by a life cycle and morphological features that are very similar to *N. americanus*, and it has been extensively used as a model for the study of human hookworm infection (4, 7, 9, 47). The analysis of the transcriptome of *N. brasiliensis* shows that more than two-thirds of the sequences with homology to sequences present in the NEMBASE database had significant similarity to *N. americanus* genes, which is in accordance with the conserved morphological features and life history of these two strongylid nematodes.

Many of the proteins predicted from the *N. brasiliensis* transcriptome contain a signal peptide, which is in accordance with findings for other helminths (27, 48); of these putative secreted proteins, a total of 6.27% (127) sequences were identified in the ESP of *N. brasiliensis*, in line with findings from other parasitic helminths (26, 27, 49, 50). However, some of the proteins (56.2%) detected in ESP did not contain a classical signal peptide, suggesting alternative secretion pathways including the presence of non-classical secretory signals or release from the parasite via exosomes (51).

The proteins present in the ESP of parasites in general, and nematodes in particular, represent the major interface between the host and the parasite, and contribute directly or indirectly to the survival or the expulsion of the parasite. Furthermore, the parasite needs to adapt to different physiological niches, depending on the developmental stage and its pattern of migration. The infective L3 stage of *N. brasiliensis* penetrates through the skin and migrates through the vasculature, whereas the adult worm lives

in a physiologically distinct niche, the gut. In the present work, we have identified 52 and 261 proteins in the ESP of the larval and adult stages of *N. brasiliensis*. In addition to this, we have shown that the proteins identified in the ESP are different to those identified in the NEx, suggesting their true secretory origin as opposed to leakage of intracellular and structural proteins from dead or damaged worms.

In our analysis of *N. brasiliensis*, only 8 proteins are detected in ESPs from both larval and adult stages, whereas in other nematodes such as *Strongyloides ratti*, more than 50% of the proteins identified in the ESP of parasitic adult females and infective larvae are common to both developmental stages. This intriguing finding suggests the need for different proteins as a consequence of adaptation to different niches; *N. brasiliensis* infective larvae and adult worms occupy distinct anatomical locations in the host, whereas *S. ratti* L3 can develop in the intestine of the host, co-habiting with adults in the same niche.

From the proteins detected in L3 ESP, two particular proteins were also highly represented in the adult secretome (Fig. 5). Both proteins had homology to GH Family 25 lysozyme 2 (GH 25 muramidase superfamily). This family of proteins has been identified at the transcript level in other hookworms (26) and in the secretome of adult *H. polygyrus* (39). In addition, in the nematode *C. elegans* and the slime mould *Dictyostelium discoideum* lysozyme has been reported to exert an antimicrobial and bacteriolytic effect, which is likely to be associated with its expression in both developmental stages (52, 53).

Other proteins that have been identified in both developmental stages include actin, a fatty-acid binding protein, two proteins of unknown function and proteases and other enzymes including cathepsin D, apyrase, acetylcholinesterase, cell death-related nuclease 7 and extracellular superoxide dismutase. Although actin is a cytoskeletal protein, it has been detected in the ESP of different helminths such as *Echinostoma caproni* or *Angiostrongylus cantonensis* (54, 55). Recently, parasitic trematodes were shown to secrete extracellular microvesicles that resembled mammalian exosomes (51), and accounts for the secretion of some proteins that lack a signal peptide. While exosome-like microvesicles have not yet been reported from parasitic nematodes, this phenomenon is entirely plausible and might explain the presence of molecules lacking a signal peptide in the ESP of *N. brasiliensis* (and most other parasitic helminths). Superoxide dismutase is an anti-oxidative protein and is involved in protection of *N. brasiliensis* and *Ancylostoma ceylanicum* against reactive oxygen species from host inflammatory cells (56). Different apyrases have been described also in the related nematode *H. polygyrus* (57), and they have been related with clot prevention in blood-feeding arthropods and nematodes (39, 58-60) and anti-coagulant. The protein acetylcholinesterase has been extensively described in *N. brasiliensis* and other nematodes and has a role in the inhibition of secretory responses by hydrolysing acetylcholine (61) and in the reduction of inflammation (62). The proteins detected in both developmental stages could be of importance in the development of future vaccines and treatments against *N. brasiliensis*, as it could reduce both the infection and the establishment of this nematode.

The most abundant larval stage specific ESPs included a metalloproteinase-inhibitor, a tyrosinase, a superoxide-dismutase and a serine protease inhibitor while, in the

adult, different ASPs, globin-like proteins, transthyretin-like proteins and vitellogenins were stage-specific. The putative metalloproteinase-inhibitor was by far the most highly represented protein in L3. It shared greatest homology with tissue inhibitor of metalloproteinase from *N. americanus* and tissue inhibitor of metalloproteinase from *A. caninum*, and belongs to the MEROPS family I35 (TIMP family). Other TIMPs have been found to be upregulated in larval stages of *N. americanus* (63) and the adult stage of *A. caninum* and *A. ceylanicum* (64, 65). Furthermore, these proteins are implicated in host-parasite relationships and modulation of the immune response (66). In contrast, the most abundant protein in adult worms was an ASP that shared sequence identity with *N. americanus* ASP-2. *Na*-ASP-2 is only expressed by hookworm L3 and not by the adult stages, and has been extensively explored as a vaccine candidate (67, 68).

ESP from both developmental stages were also categorized according to the presence of conserved (Pfam) domains. The most abundantly represented family of proteins in the L3 ESP was the astacin metalloproteases (Peptidase family M12A (PF01400)). In eukaryotes, astacins adopt a range of functions, including the activation of growth factors, degradation of polypeptides and the processing of extracellular proteins (69). Astacins have been found in other free-living and parasitic nematodes (50, 70-72) including the canine hookworm *A. caninum* (73); *A. caninum* L3 secrete an astacin which digests connective tissues upon invasion of the host skin, and the protein has shown potential as a hookworm vaccine (73, 74). *S. stercoralis* strongylastacin is only expressed by L3, and it is thought to be involved in the invasion process (75). Other astacins such as NAS-36 and NAS-37 from the free-living nematode *C. elegans*, play important roles in cuticle ecdysis during moulting (71, 76), while NAS-36

homologues from the parasitic nematodes *H. contortus* and *B. malayi* play important functions in parasite development (77, 78). A phylogenetic analysis of astacins from *N. brasiliensis* and other nematodes revealed a monophyletic clade formed exclusively by selected sequences from adult *N. brasiliensis* ESP (i.e. m72921, m440287, m440290) and homologues from the predicted proteome of *N. americanus* and the free-living nematode *C. brenneri*, to the exclusion of sequences from all other nematode species included in the analysis, as well as other hookworm homologues (Fig. 6). The fact that, to the best of our knowledge, some members of the (parasite) astacin family are shared exclusively between *N. americanus* and *N. brasiliensis* raises questions about the specific roles that these proteins may play in the biology of these hookworm species, and serves to highlight the biological similarities between the human and the rat hookworm.

In contrast, the most abundant family of proteins in the adult ESP was the SCP/TAPS (PF00188). The abundance of this subfamily of proteins was also confirmed by the 2D gel analysis, where almost 70% of the spots identified presented a single or double CAP motif. We identified more than 30 SCP/TAPS proteins in the ESP of L3 and adult stages from *N. brasiliensis*, which is in accordance with the diversity of this group of proteins in most nematodes (57), most notably hookworms, where they constitute more than 30% of the adult worm ESP (26, 79). Most of the SCP/TAPS proteins from *N. brasiliensis* were secreted only by adult worms, and only 3 proteins were detected from L3 ESP, and one protein (m259086) was common to the ESP of both developmental stages. SCP/TAPS proteins with CAP motifs are generally abundant in helminth secretomes, and while at least two members from parasitic nematodes appear to have immunomodulatory properties (80, 81), relatively little

functional information is available despite the recent advances in the structural biology of this protein family (82). Due to the relative ease of genetic manipulation of *C. elegans* compared to parasitic nematodes, additional functional roles for SCP/TAPS proteins have been identified, including antimicrobial activity, normal body formation and lifespan (83-85), highlighting the extensive array of functions that this intriguing protein family has adopted.

The phylogenetic analysis of SCP/TAPS proteins revealed that, while the vast majority of hookworm SCP/TAPS clustered together with homologues from other parasitic nematodes with varying bootstrap/posterior probabilities support (cf. Fig. 7), a small subset of sequences from the rat and human hookworms formed a separate clade (cf. Fig. 7), thus leading to the hypothesis that, within the SCP/TAPS family, these members might have evolved biological functions specific to *N. brasiliensis* and *N. americanus* only, and distinct from their homologous counterparts

To assess the overall sequence similarities between the secretomes of *N. brasiliensis* and related nematodes, we performed a similarity analysis using ESP from *N. brasiliensis*, *H. polygyrus*, *B. malayi* and *N. americanus*. This type of analysis plots in a two-dimensional scale the relative similarities of protein sequences between one species (*N. americanus*) and three comparators as described previously for other nematodes (17, 37). As highlighted in Fig. 8, *N. americanus* ESP had more homologues in the ESP from *N. brasiliensis* than from either *H. polygyrus* or *B. malayi*. We selected these species as comparators because they can complete their life cycles in rodents and their ESP had been characterised using proteomics techniques (as opposed to predicted using bioinformatics tools). We believe that the sequence

identities between the secretomes of *N. brasiliensis* and *N. americanus* support the morphological and developmental similarities between these two species, and lends strong support for the use of *N. brasiliensis* as a model for human hookworm disease (7, 9, 39, 47).

This is the first study that provides an in-depth overview of the proteins secreted by *N. brasiliensis*, which could be of importance in unravelling the molecular architecture of a complex host-parasite relationship. Our data lends strong molecular support for the use of *N. brasiliensis* as a robust animal model for human hookworm infection, and reveals groups of proteins that have expanded to play critical roles in the biology of migrating larvae and establishment, feeding and modulation of immunity by the adult worm in the host gut. The ESP described herein present potential targets for the development of new drugs and vaccines against gastrointestinal nematodes of humans and animals, and might also hold promise as a novel approach to control autoimmune and allergic inflammatory disorders (86) that are increasing at an alarming rate in developed countries.

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Footnotes

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Figure legends

FIG. 1. *Silver stained analytical 2-DE gel of N. brasiliensis adult excretory/secretory proteins.* Numbered circles indicate the spots that were analysed by mass spectrometry. Annotation of the individual spots can be found in Table 1.

FIG 2. *Overlap between L3 and adult excretory/secretory proteins.* A. Venn diagram showing the common proteins between L3 ESP, adult ESP and adult NEx *N. brasiliensis*. Abbreviations used: ESP- excretory/secretory products from *N. brasiliensis*; NEx- whole worm extract from *N. brasiliensis*. B. 2D-DIGE image comparing L3 ESP (green spots) and adult ESP (red spots) from *N. brasiliensis*.

FIG 3. *Gene Ontology analysis.* Bar graph showing the most abundantly represented gene ontology molecular function terms in excretory/secretory proteins derived from *N. brasiliensis* L3 (A) and adult (B).

FIG 4. *Pfam analysis.* Bar graph showing the most represented protein families (Pfam) in the excretory/secretory proteins of *N. brasiliensis* L3 (A) and adult (B), the predicted proteome from L3 (C) and adult (D), and the predicted proteome containing a signal peptide from L3 (E) and adult (F). Coloured bars represent the families present in more than two panels.

FIG 5. *Comparison of the protein abundance in the L3 excretory/secretory proteins (ESP), adult ESP and adult adult worm extract (NEx) samples.* A. The 50 most abundant proteins in L3 ESP ranked by total spectrum count (left) compared to the

same proteins in adult ESP (centre) and adult NEx (right). Maximum values are coloured in red and minimum in blue. *B.* The 50 most abundant proteins in adult ESP ranked by total spectrum count (centre) compared to the same proteins in L3 ESP (left) and adult NEx (right). The 50 most abundant proteins in adult NEx ranked by total spectrum count (right) compared to the same proteins in L3 ESP (left) and adult ESP (centre).

FIG 6. *Phylogenetic relationships of astacins based on Bayesian Inference (BI).* The posterior probability supporting each clade is indicated. The accession numbers of each protein are shown.

FIG 7. *Phylogenetic relationships of SCP/TAPS proteins based on Bayesian Inference (BI).* The posterior probability supporting each clade is indicated. The accession numbers of each protein are shown.

FIG 8. *Similarity analysis.* Relationships of ESP from *Brugia malayi*, *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* were compared to the ESP predicted from the genome of *Necator americanus* and displayed in a Simitri plot (37).

TABLE 1.- *Proteins identified in the 2D-PAGE of Nippostrongylus brasiliensis using MASCOT search engine. Asterisks (*) represent proteins identified by LC-MS/MS. Abbreviations used: NP- number of peptides; SP- signal peptide; TD- transmembrane domain; GS- glycosylation site. Individual ions scores > 38 indicate identity or extensive homology (p<0.05). ? represents sequences where absence of signal peptide could not be confirmed.*

Spot number	Protein Number	Mascot score	MW (exp/theo)	pI (exp/theo)	NP	Blast annotation	Species	SP	TD	GS	Domain
1	m. 127459	104	94.73/42.6	4.7/4.69	1	sp P10736 Venom allergen_5.01	Dolichovespula	Y	N	Y	Double SCP
2	m. 127468	96	94.74/47.50	4.9/8.95	1	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
3*	m.127554	1003	85.89/49.91	5.7/4.91	5	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	Y	N	Y	Double SCP
4	m. 127459	114	83.15/42.60	6/4.69	1	sp P10736 Venom allergen_5.01	Dolichovespula	Y	N	Y	Double SCP
5*	m.282979	1515	80.50/22.10	6.35/9.3	6	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	?	N	Y	Single SCP
6*	m.130034	980	75.50/23.36	6.85/9.31	9	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	?	N	Y	Single SCP
7	m.130034	201	73.13/23.48	7.25/9.31	5	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Single SCP
8	m.130034	136	70.85/23.48	7.6/9.31	3	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Single SCP
9	m.130034	217	70.85/23.48	8/9.31	3	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Single SCP
10	-	-	-	-	-	-	-	-	-	-	-
11	m.36493	91	85.89/20.64	8.35/9.37	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	N	Single SCP
12	m.210487	255	77.96/49.81	7.9/6.2	4	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
13*	m.68479	55	216.54/19.46	9.5/9.74	9	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	N	Single SCP
14	m.50190	91	77.96/25.03	9.05/9.38	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
15	m.50190	97	64.49/25.03	9/9.38	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
16*	m.210487	524	53.73/49.81	8.05/6.2	4	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
17*	m.210487	766	53.73/49.81	8.75/6.2	4	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
18	m.210487	125	53.73/49.81	7.95/6.2	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
19	m.210487	213	53.73/49.81	7.6/6.2	4	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
20*	m.210487	587	53.73/49.81	7.4/6.2	4	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP

Secretome of *Nippostrongylus brasiliensis*

21*	m.394113	463	68.65/36.21	6/5.01	6	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	?	N	Y	Single SCP
22	m.280800	87	70.85/64.96	4.45/4.74	1	Acetylcholinesterase 4	Caenorhabditis	Y	N	Y	Esterase lipase
23*	m.425554	1566	47.81/94.55	4.5/5.43	11	sp Q18673 Neprilysin-1	Caenorhabditis	N	Y	Y	Gluzincin superfamily
24	m.271310	74	49.20/49.04	6.7/6.95	1	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	Y	Double SCP
25*	m.210487	274	53.73/49.81	6.95/6.2	3	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
26	m.271310	81	47.81/49.04	7.3/6.95	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	Y	Double SCP
27*	m.271310	888	46.46/49.04	8/6.95	8	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	Y	Double SCP
28	m.209558	188	40.47/40.83	9.35/8.7	3	sp Q16937 Ancylostoma secreted protein	Ancylostoma	?	N	Y	Double SCP
29*	m.21970	1193	33.83/95.10	8.8/6.6	10	sp O16796 Neprilysin-2	Caenorhabditis	N	Y	Y	Gluzincin superfamily
30*	m.161819	1467	52.16/58.36	7.95/8.79	16	sp Q7JL11 Zinc_metalloproteinase_nas-31	Caenorhabditis	Y	Y	Y	Astacin
31	m.223466	97	42.73/46.89	7/6	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	Y	Double SCP
32	m.285697	109	43.93/46.27	6.6/5.56	2	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	N	DUF Superfamily
33*	m.418883	1492	33.83/52.11	4.95/7.24	12	sp O93428 Cathepsin_D	Chionodraco	N	N	Y	Asp
34	-	-	-	-	-	-	-	-	-	-	-
35*	m.55433	980	33.83/43.33	6.6/6.32	10	sp P46563 Fructose-bisphosphate_aldolase_2	Caenorhabditis	Y	N	N	Aldolase
36*	m.55433	1603	33.83/43.33	8.75/6.32	13	sp P46563 Fructose-bisphosphate_aldolase_2	Caenorhabditis	Y	N	N	Aldolase
37	Keratin	508	27.01/65.98	9.35/8.16	7	-	Homo sapiens	-	-	-	-
38*	m.127462	577	27.57/48.65	8.35/8.39	3	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	Y	N	Y	Double SCP
39*	m.233248	2323	23.20/25.54	4.5/5.32	13	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	N	Single SCP
40	m.122208	330	21.71/28.30	4.6/5.05	5	sp Q86KC1 Probable_GH_family_25_lysozyme	Dictyostelium	Y	N		GH25_muramidase
41	m.233248	223	23.20/25.54	4.9/5.32	5	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	N	Single SCP
42	m.233248	88	23.61/25.54	5.25/5.32	1	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	N	Single SCP
43	m.233248	61	23.61/25.54	5.45/5.32	1	sp Q16937 Ancylostoma secreted protein	Ancylostoma	Y	N	N	Single SCP
44	m.233245	163	22.06/19.40	6.25/7.23	4	sp B2MVK7 Venom_allergen_5	Rhynchium	Y	N	Y	Single SCP
45	m.233245	121	22.06/19.40	6.5/7.23	2	sp B2MVK7 Venom_allergen_5	Rhynchium	Y	N	Y	Single SCP
46	m.233245	80	22.06/19.40	6.7/7.23	3	sp B2MVK7 Venom_allergen_5	Rhynchium	Y	N	Y	Single SCP
47*	m.37549	1846	21.37/21.10	7.65/8.05	10	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	?	N	N	Single SCP
48*	m.233245	999	52.16/19.40	8.75/7.23	6	sp B2MVK7 Venom_allergen_5	Rhynchium	Y	N	Y	Single SCP
49*	m.37549	1544	20.44/21.10	8.4/8.05	8	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	?	N	N	Single SCP
50	m.433945	90	17.83/19.76	9.35/9.38	2	Globin	Nippostrongylus	Y	N	Y	Globin

Secretome of *Nippostrongylus brasiliensis*

51*	m.229449	782	17.30/19.70	8.85/6.59	3	sp P51535 Myoglobin	Nippostrongylus	Y	N	N	Globin
52*	m.417418	2990	16.83/20.32	8.2/6.32	6	sp P48817 Nucleoside_diphosphate_kinase	Nippostrongylus	N	N	N	kinase
53*	m.229449	539	17.73/19.70	8.05/6.59	4	sp P51535 Myoglobin	Nippostrongylus	Y	N	N	Globin
54*	m.123267	595	19.87/22.04	6.3/6.9	8	sp Q9NL98 Peroxiredoxin	Ascaris	N	N	N	Thioredoxin
55*	m.285697	2022	45.17/46.15	6/5.56	11	sp Q16937 Ancylostoma_secreted_protein	Ancylostoma	Y	N	N	DUF Superfamily
56*	m.211569	960	42.16/47.10	8.75/6.61	14	sp Q27527 Enolase	Caenorhabditis	N	N	Y	Enolase

LEGENDS TO SUPPLEMENTAL MATERIAL

FIG. S1. *Gene Ontology*. Gene Ontology terms relating to biological process (A, D), molecular function (B, E) and cellular component (C, F) assigned to the excretory/secretory proteins from L3 and adult from *N. brasiliensis*, respectively.

TABLE SI. *Proteins identified in the 2D-PAGE of Nippostrongylus brasiliensis using MASCOT search engine*. Asterisks (*) represent proteins identified by LC-MS/MS. Individual ions scores > 38 indicate identity or extensive homology ($p < 0.05$).

TABLE SII. *Scaffold generated protein report of L3 ESP from N. brasiliensis*.

TABLE SIII. *Scaffold generated peptide report of L3 ESP from N. brasiliensis*.

TABLE SIV. *Scaffold generated protein report of adult ESP from N. brasiliensis*.

TABLE SV. *Scaffold generated peptide report of adult ESP from N. brasiliensis*.

TABLE SVI. *Scaffold generated protein report of adult NEx from N. brasiliensis*.

TABLE SVII. *Scaffold generated peptide report of adult NEx from N. brasiliensis*.

TABLE SVIII. *Single peptide identifications from 2D analysis*.

Figure 1

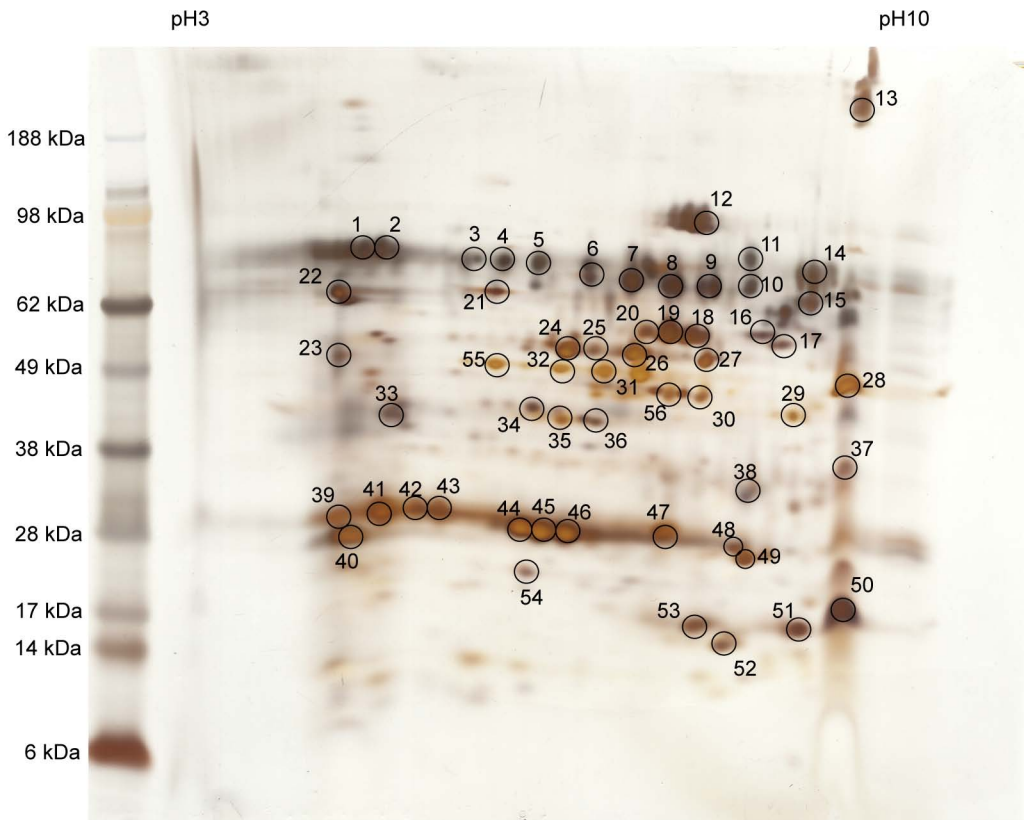
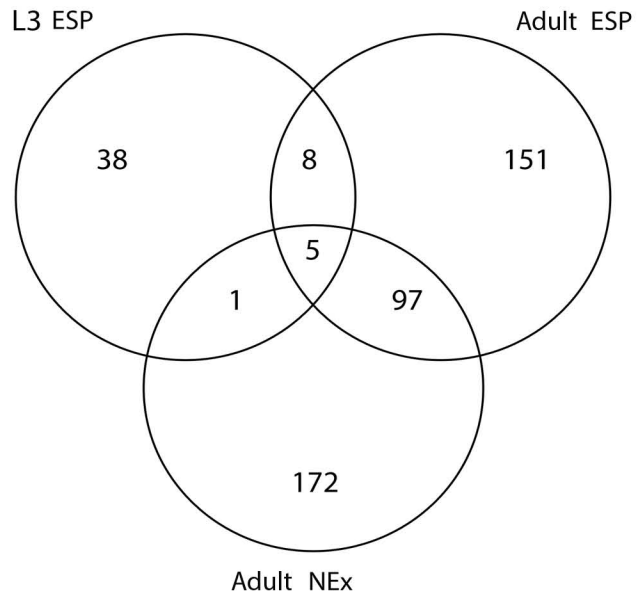


Figure 2

A



B

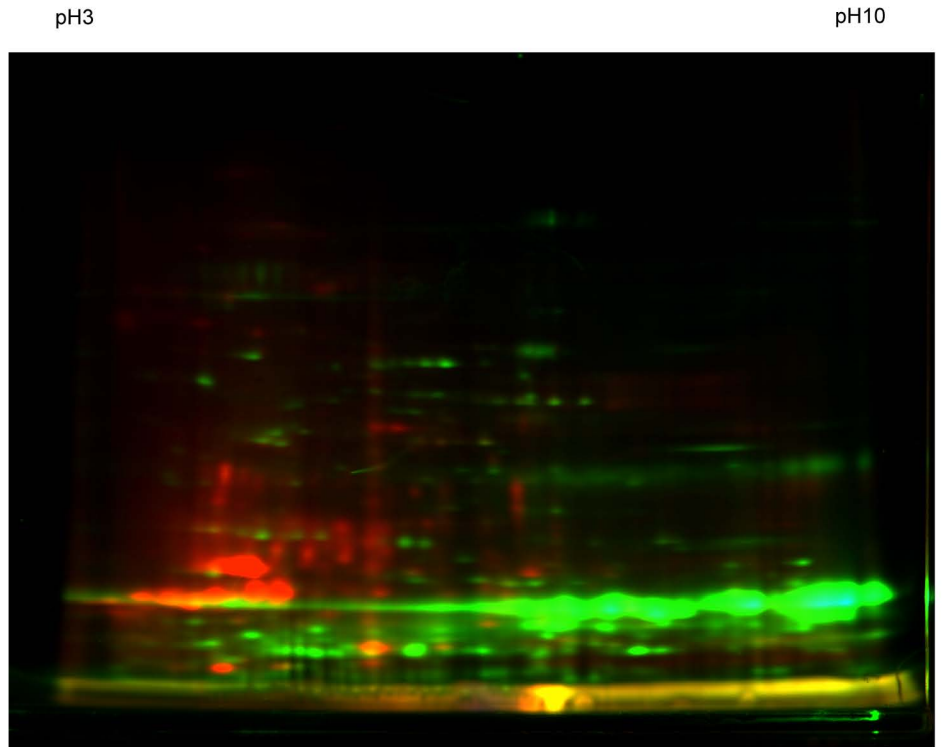
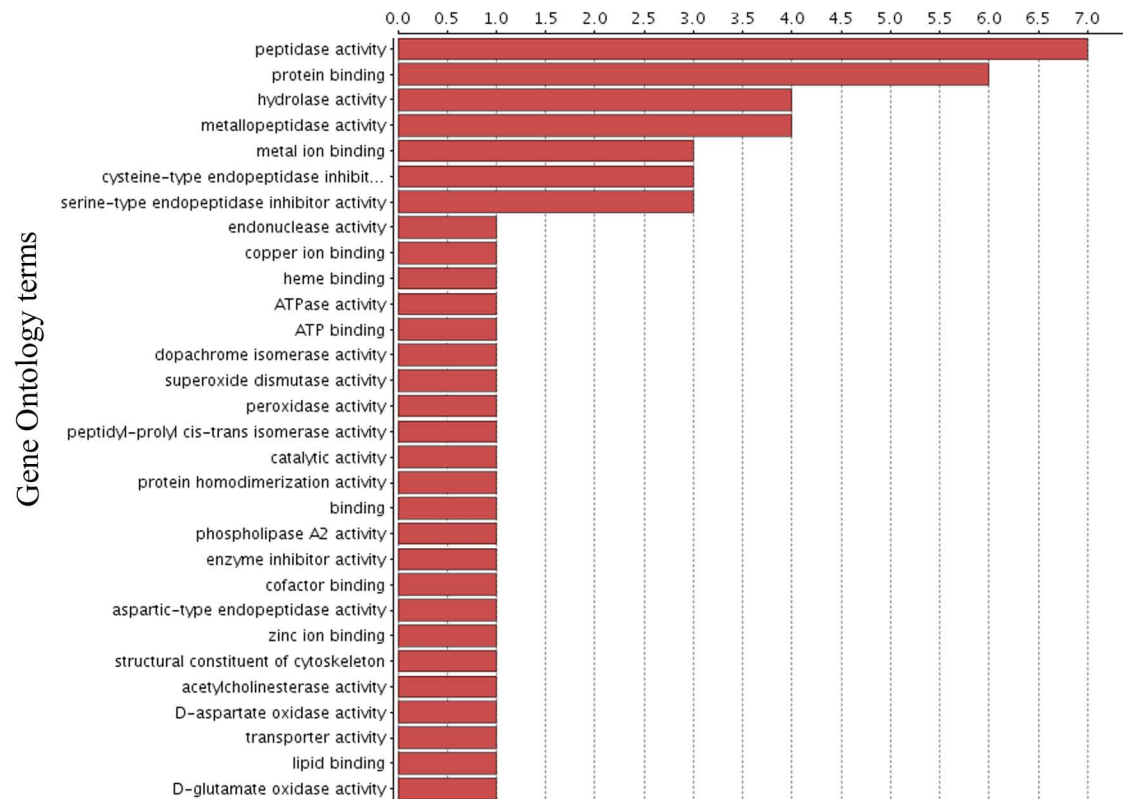


Figure 3

A

Sequences



B

Sequences

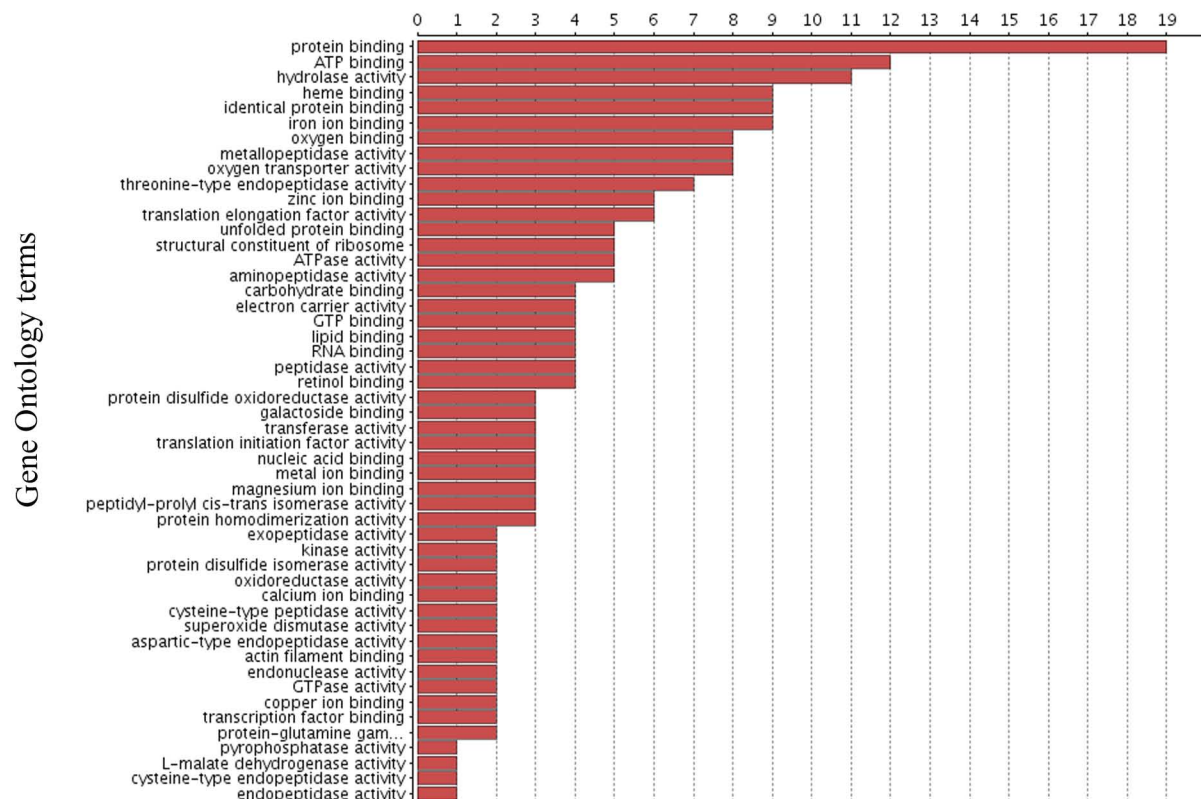
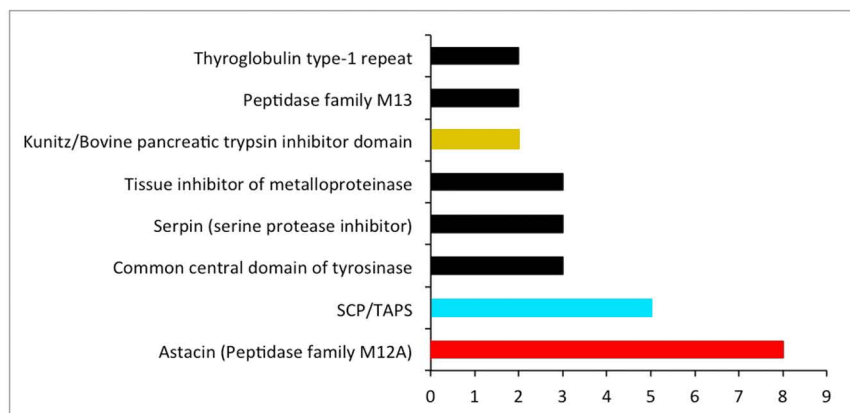
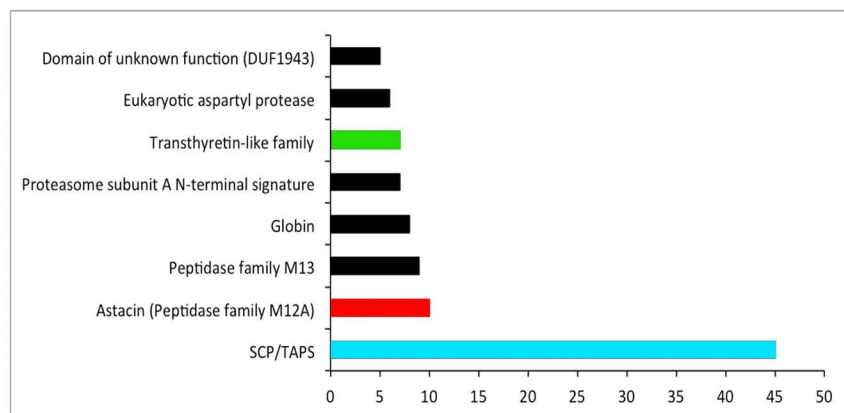


Figure 4

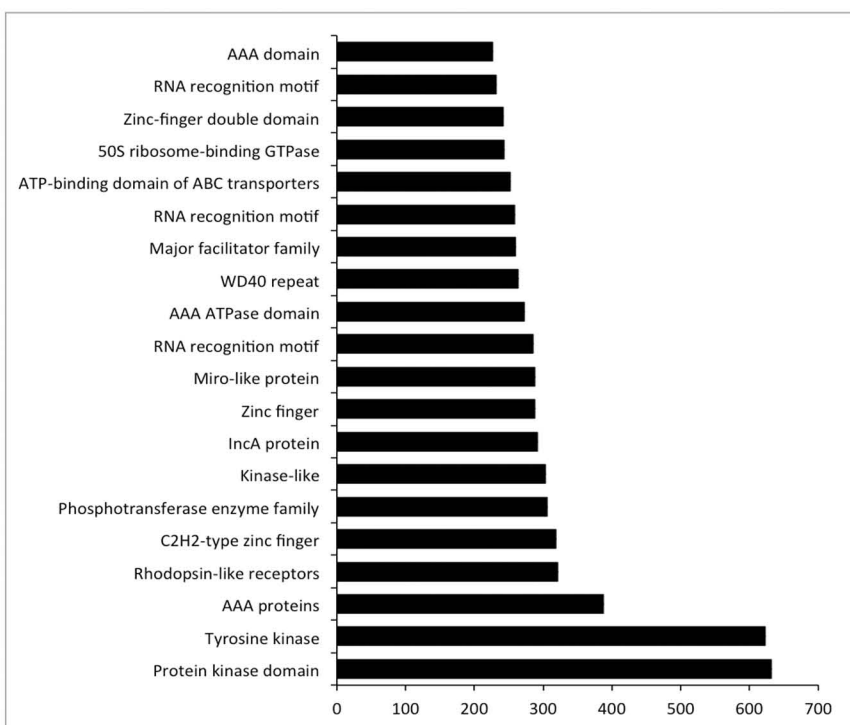
A



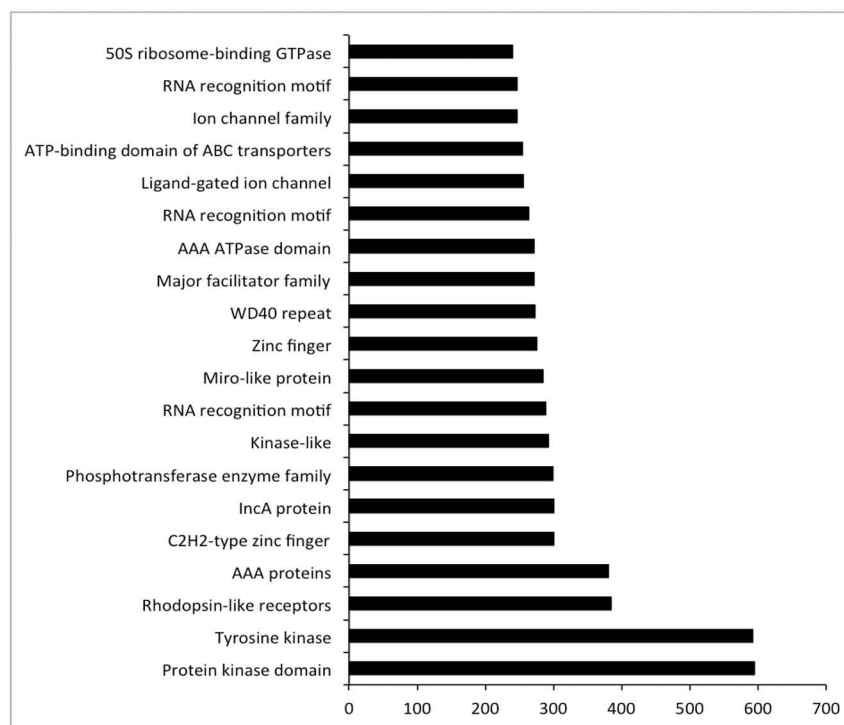
B



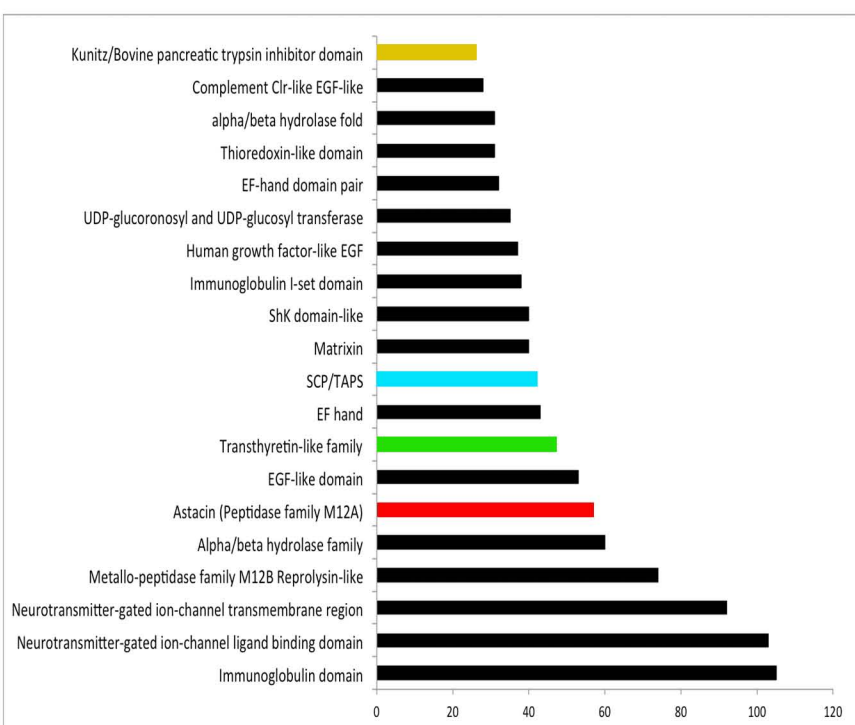
C



D



E



F

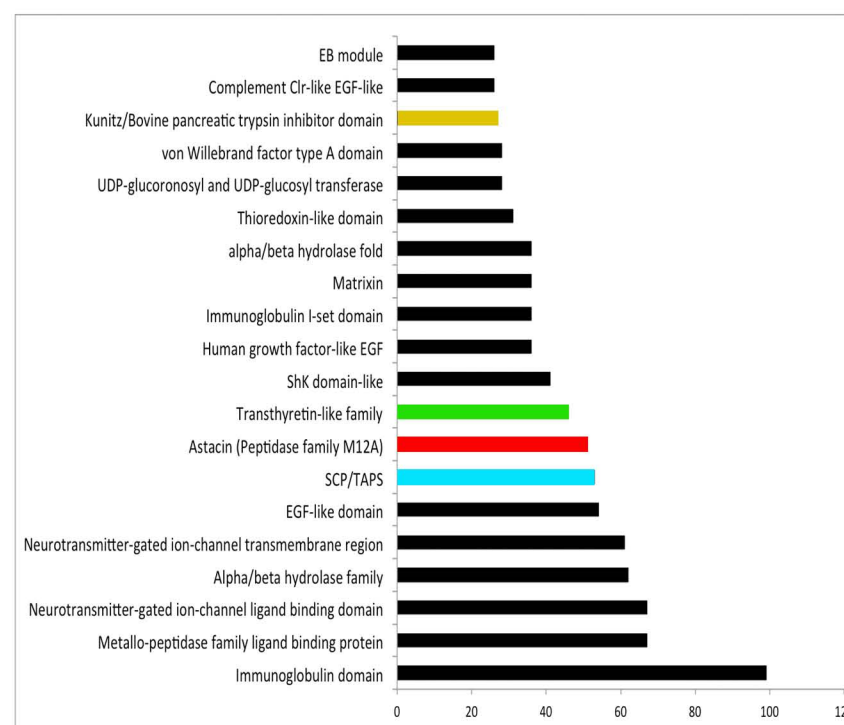
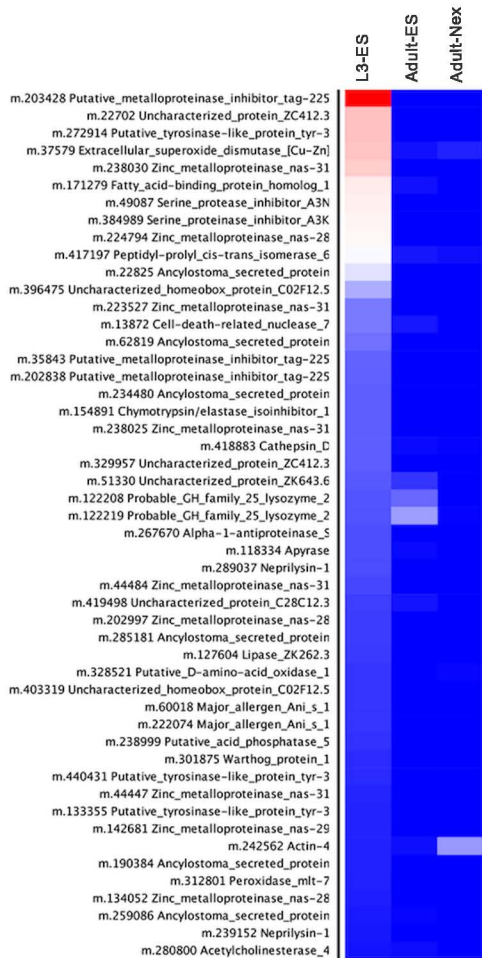
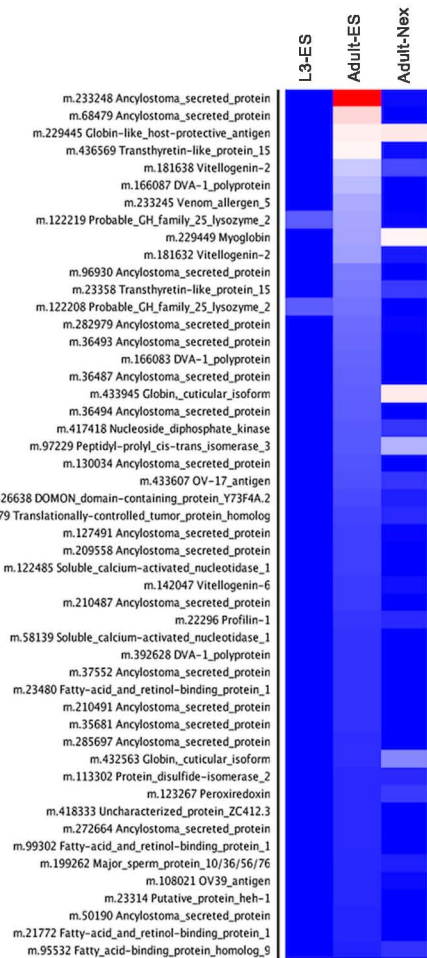


Figure 5

A



B



C

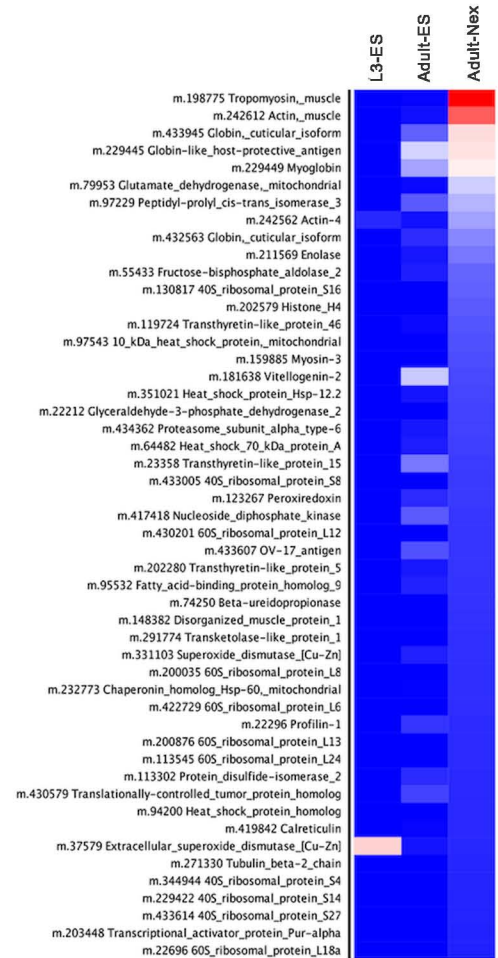
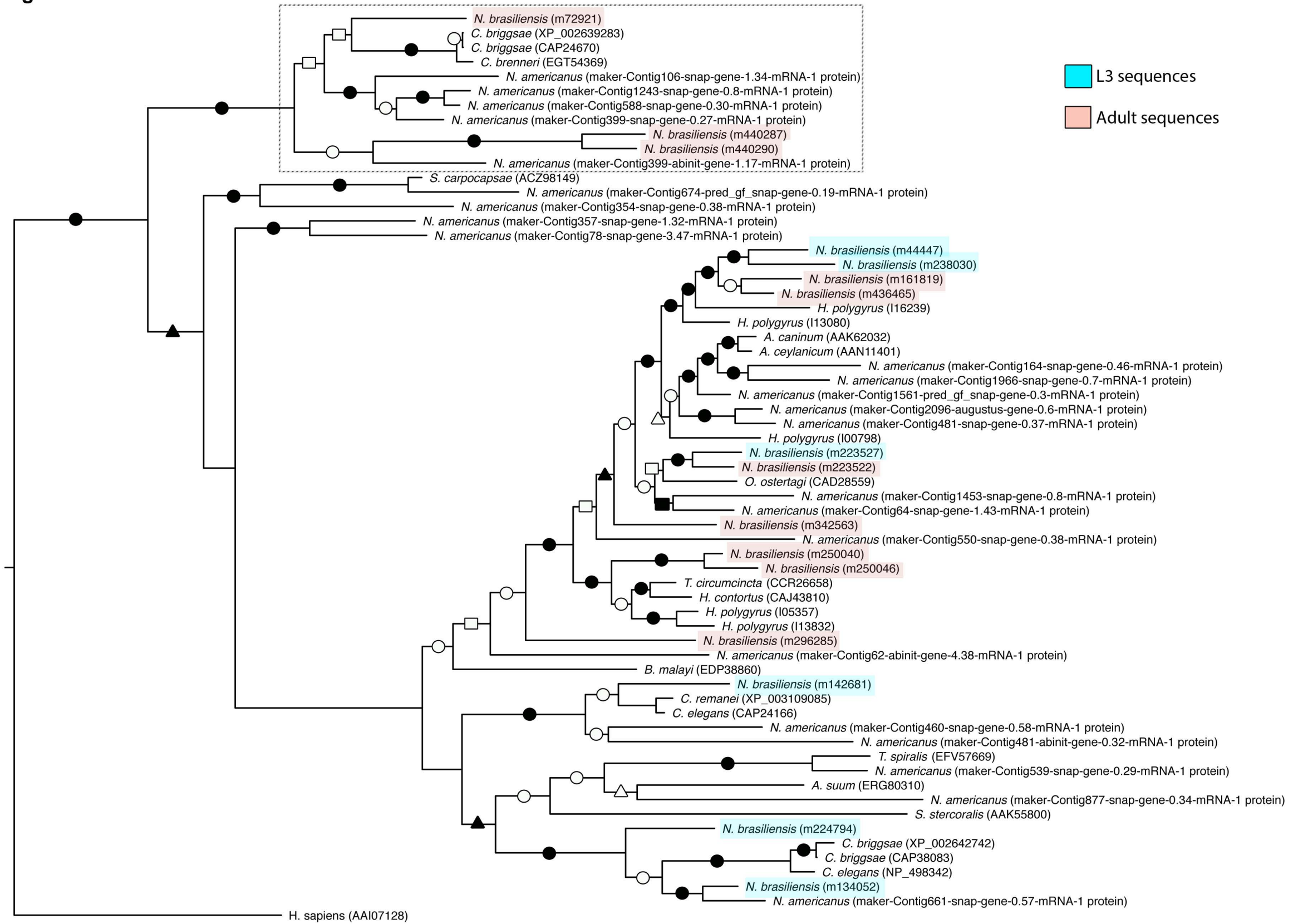


Figure 6



● 1.00

○ 0.90-0.99

■ 0.80-0.89

□ 0.70-0.79

▲ 0.60-0.69

△ 0.50-0.59

Figure 7



● 1.00

○ 0.90-0.99

■ 0.80-0.89

□ 0.70-0.79

▲ 0.60-0.69

△ 0.50-0.59

Figure 8

