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Research paper

Secretome of the carcinogenic helminth *Spirocerca lupi* reveals specific parasite proteins associated with its different life stages

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ABSTRACT

Spirocerca lupi is a parasitic and carcinogenic nematode of canids distributed in tropical and subtropical regions around the world. The excretion-secretion proteins (PES) of *S. lupi* have been suggested to play a role in the pathogenesis of its infection. We aimed to identify the PES of different stages of *S. lupi* and search for proteins that would be useful for diagnostic, therapeutic and vaccination purposes as well as understand their functions. A nano-UPLC mass spectrometry *de novo* analysis was performed on proteins collected from cultures of *S. lupi* L3 larvae, L4 females, adult females and adult males from naturally infected hosts. A total of 211 proteins were identified in all cultures. Accordingly, 117, 130, 99 and 116 proteins were detected in L3 larva, L4 females, adult females and adult males, respectively, with a strong correlation in the biological replicates (Pearson coefficients > 0.73). Fourty-four proteins were detected in all developmental stages, 64 were stage-specific and 49 were exclusively identified in L4 females. Cell compartment enrichment analysis revealed that proteins common to all stages were cytoplasmatic ($p < 9.x10^{-6}$), whereas L4 unique proteins were in collagen trimers, and macromolecular complexes ($p < 0.00001$). Functional enrichment analysis of proteins showed significant enrichment in lipid metabolism in L3-unique proteins ($p < 0.00005$), in mannose metabolism and protein deglycosylation for L4-unique proteins ($p < 0.00004$), and in phosphorus metabolism in proteins shared by all stages ($p < 2.1\ x10^{-9}$). Interestingly, annexin 6, associated with cancer in humans, was detected in all life stages, but in a larger abundance in L4 females and adults. These findings indicate that *S. lupi* establishes complex interactions with its hosts by an arsenal of proteins expressed in different patterns in each life stage which influence the pathogenesis and oncogenesis of *S. lupi* and may be used as potential targets for diagnostic assays, drug targets or vaccine candidates.

1. Introduction

Spirocerca lupi is a parasitic nematode of canids prevalent in tropical and subtropical regions around the world which causes a severe and potentially life-threatening disease known as spirocercosis. The life cycle of this worm involves a coprophagous dung beetle and a canid as intermediate and definitive hosts, respectively (van der Merwe et al., 2008). In the beetles, eggs hatch under specific physical and chemical conditions (Rojas et al., 2017) to release L1 larvae that further develop to the third larval stage, L3, which is infective to the vertebrate host. *S. lupi* adults usually develop in nodules which protrude into the esophageal lumen, causing vomiting and regurgitation (Mazaki-Tovi et al., 2002). Moreover, cases of aberrant migration have been reported with *S. lupi* migrating through the thoracic cavity (Dvir et al., 2001; Fischer and Carneiro, 1974; Garg et al., 1989; Harrus et al., 1996; Stephens et al., 1983), urinary system (Thanikachalam et al., 1984), subcutaneous tissues (Harrus et al., 1996), gastrointestinal tract (Georgi et al., 1980) and the spinal cord (Chai et al., 2018; Dvir et al., 2007). Thus, the clinical signs of spirocercosis can vary greatly according to the progression of the disease, anatomic locations of the worms and additional complications such as rupture of aneurisms in the aorta where worm migration has taken place (van der Merwe et al., 2008). Importantly, infection can advance to the malignant transformation of the esophageal nodules into fibrosarcomas or osteosarcomas (Dvir et al., 2008), associated with hypertrophic osteopathy and metastatic dissemination. The pathogenesis of spirocercosis and its association with malignant transformation is not well understood and it has been hypothesized that the latter results from molecules potentially secreted by *S. lupi* (Dvir et al., 2010; Herrera and Ostrosky-Wegman, 2001; Meléndez and Suárez-Pellín, 2001).

Products of excretion-secretion (PES) are molecules exported by active secretory pathways or excretion of waste products into the host

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environment (Harnett, 2014). In helminths, PES have a wide range of activities, which include structural and metabolic components of the worm, host invasion, host cell proliferation and immune modulation molecules (Hewitson et al., 2009). Overall, they attest of the presence of complex and dynamic interactions between parasites and their hosts (Cuesta-Astroz et al., 2017). PES have been associated with the pathogenesis of helminth infections and can be recognized by the host's immune system, thus, PES have been proposed as potential vaccine candidates and as diagnostic antigens (Harnett, 2014; Sotillo et al., 2017). Furthermore, PES have been demonstrated to be useful as vaccine and diagnostic antigens as well as drug targets for several worm species of human and veterinary importance (Mehrdana and Buchmann, 2017; Nisbet et al., 2013). Vaccination using galectins (Yanming et al., 2007) and glyceraldehyde 3-phosphate dehydrogenase (Han et al., 2012) of *Haemonchus contortus* has decreased the worm burden in challenged animals, the use of recombinant PES of *Toxocara canis* have been used for diagnosing human infections (Anderson et al., 2015), and peptidase inhibitors have been used to decrease the activity of metallopeptidases of *Schistosoma mansoni* (Dvorak et al., 2016). Only one study has attempted to characterize the PES of *S. lupi* so far (Sako et al., 2017). However, while in cultivated media from other nematodes, about 100 to 200 proteins have been identified, only nine proteins were identified in the previous study (Sako et al., 2017). Therefore, the proteomic profile of *S. lupi* PES remains unclear.

It has been suggested that *S. lupi´s* PES actively influence the pathogenesis of the infection (Dvir et al., 2010). Thus, the aim of the present study was to identify and functionally analyze the PES of different stages of *S. lupi* maintained under *in vitro* conditions with available helminth databases. For this purpose, we performed a bottom-up *de novo* proteomic analysis of the excreted-secreted proteins of *S. lupi* L3 larvae, L4 females, adult males and adult females collected from naturally-infected dogs and dung beetles and maintained under *in vitro* conditions. More than 1400 peptides belonging to 211 different proteins homologous to sequences of other nematodes were obtained. These proteins are involved in structural and metabolic functions, as well as host invasion and immune regulation. Remarkably, a differential protein expression was obtained between developmental stages, mainly in the secretion of extracellular-matrix remodeling and interacting molecules by the L4 immature females, and in proteins related to lipid metabolism in L3 larvae. In addition, the potentially pro-oncogenic protein annexin 6 was found in the adult and L4 stages, suggesting a possible role in malignant transformation occurring in spirocercosis. These findings shed light on the molecular host-parasite interplay and pathogenesis of spirocercosis.

2. Materials and methods

2.1. Collection and incubation of S. lupi L3 larvae

Onthophagus sellatus dung beetles were collected from a public park in Central Tel Aviv. The beetles were maintained in the laboratory according to the conditions described elsewhere (Rojas et al., 2017) and dissected for the search of *S. lupi* larval stages (Fig. 1A). The larvae found were immediately placed in RPMI-1640 medium supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin, 0.2 mg/ml gentamycin, 0.25 μg/ml amphotericin, 2% D-glucose and 10 mM L-glutamine (referred to as supplemented RPMI) and serially washed 10 times. Then, the larvae were placed in sterile 24-well cell culture plates with 2 ml of supplemented RPMI medium and incubated at 26 °C with 5% CO_2 (Fig. 1B and Supplementary Video. S1). The media was discarded after 24 of incubation to remove potentially host-associated proteins, and new medium was added to the larvae. These stages were incubated for 24 additional hours and the media from two different L3 larvae (i.e. biological replicates) was collected for protein analysis. Subsequently, fetal bovine serum (Biological Industries LTD, Kibbutz Beit-Haemek, Israel) was added to a final concentration of 20% to the supplemented RPMI and larvae were grown at 37 °C with 5% of CO_2 for 140 days. Their growth was assessed by measuring the larvae's body length from day 56 to day 140 using the ImageJ 1.48v tool (Supplementary Fig. S1a) (Schneider et al., 2012), to assess their development and potential molting to L4 stages. During all the observation period the worms remained viable, without bacterial or fungal contamination and no morphological signs of molting were observed.

2.2. Collection and incubation of S. lupi adult worms

S. lupi adult female and male specimens were collected during post-mortem dissection of naturally-infected dogs with non-neoplastic esophageal nodules. In addition, *S. lupi* L4 immature females were obtained from a naturally-infected dog with aortic aneurisms related to the infection (Fig. 1C and D). On microscopic observation, the L4 specimens did not present any eggs in their uterus, and the vulva opening was incompletely formed. After collection, all worms were immediately placed in PBS with 200 U/ml penicillin and 0.2 mg/ml streptomycin for transportation to the laboratory. Then, the worms were serially washed ten times in supplemented PBS for 10 min each wash to remove any potential bacterial contamination. The worms were incubated at 37 °C with 5% CO_2 in sterile 6-well cell culture dishes with 5 ml of supplemented RPMI, as described previously (Rojas et al., 2017) (Supplementary Video. S2). L4 females, adult females and adult males were each placed individually in single wells. In addition, a set of adult males and females were arranged as couples in shared wells. After 24 h, the media was discarded to eliminate potential dog-associated proteins and new medium was added to the worms. The media were monitored for gross bacterial contamination by checking the media's turbidity, change in the pH and microscopic observation. The worms were incubated for an additional 24 h, and the media of two different worms grown individually per stage (i.e. biological replicates) was collected for protein analysis. The L4 females, adult females and adult males were incubated at the above conditions for 5, 36 and 16 days, respectively, free of bacterial and fungal contamination (Supplementary Fig. S1B).

2.3. Treatment of media

The media of L3 larvae, L4 females and adults were filtered in 0.22 μm-pore filters immediately after collection, concentrated in 10 kDa molecular weight-exclusion pore filter units (MilliporeSigma, Burlington, Massachusetts, USA) at 4000 *x* g for 30 min at 4 °C and dialyzed against PBS at 4000 *x* g for 30 min at 4 °C. Aliquots of the concentrated and dialyzed media were stored at −80 °C until analysis. Protein concentration was determined using the BCA assay according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.4. Protein separation and mass spectrometry analyses

The samples were subjected to in-solution tryptic digestion, followed by a desalting step. Proteins in the samples were first denatured using a 5% sodium duodecyl sulfate (SDS) solution, then reduced by incubation with 5 mM dithiothreitol (Sigma-Aldrich Corporation, St Louis, Missouri, USA) for 30 min at 60 °C and alkylated with 10 mM iodoacetamide (Sigma-Aldrich Corporation, St Louis, Missouri, USA) protected from light for 30 min at 21 °C. After this, each sample was loaded into S-trap micro-columns (ProTiFi LLC, Huntington, New York, USA) according to the manufacturer's instructions. Briefly, after loading the samples they were washed with 90%:10% methanol:50 mM ammonium bicarbonate. Samples were then incubated with trypsin (1:50 trypsin: protein) for 1.5 h at 47 °C. The digested peptides were eluted using 50 mM ammonium bicarbonate, and trypsin was added again to this fraction and incubated with it overnight at 37 °C. Two more elution steps were made using 0.2% formic acid and 0.2% formic acid in 50% acetonitrile. The three eluted solutions were pooled together, and

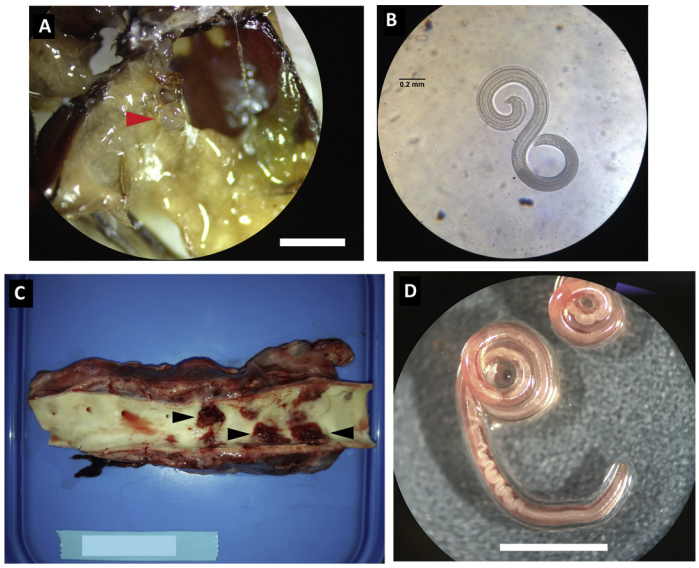

Fig. 1. *Spirocerca lupi* L3 larvae and L4 females obtained from naturally infected hosts. L3 larvae were obtained from infected *Onthophagus sellatus* dung beetles (A) and maintained *in vitro* for the collection of their excretory-secretory proteins (B). L4 females were collected from aortic aneurisms of an infected dog (C) and their morphology and sexual maturation confirmed by light microscopy (D). Scale bars in a, b and d represent 1 cm, 0.2 mm and 300 µm, respectively.

vacuum centrifuged to dryness. Samples were kept in −80 °C until analysis.

Each sample was loaded using split-less nano-Ultra Performance Liquid Chromatography (nano-UPLC) at 10 kpsi (nanoAcquity, Waters, Milford, Massachusetts, USA). The mobile phase consisted of Buffer A: H_2O + 0.1% formic acid, and Buffer B: acetonitrile + 0.1% formic acid. Dried digested samples were resuspended in 97:3% H_2O: Buffer B. Desalting of the samples was performed online using a reversed-phase Symmetry C18 trapping column of 180 µm internal diameter, 20 mm length and 5 µm particle size (Waters, Milford, Massachusetts, USA). The peptides in the samples were separated using a C18 HSS T3 nano-column of 75 µm internal diameter, 250 mm length and 1.8 µm particle size (Waters, Milford, Massachusetts, USA) at 0.35 µl/min while maintaining a column temperature of 45 °C. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 25% of Buffer B in 155 min, 25% to 90% Buffer B in 5 min, kept at 90% for 5 min and then back to initial conditions. ULC/MS grade solvents were used for all chromatographic steps.

The nano-UPLC was coupled online to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) through a nanoESI emitter (10 µm tip; New Objective; Woburn, Massachusetts, USA) using a FlexIon nanospray apparatus (Proxeon) with a spray voltage of 2.0 kV. Data was acquired in the data dependent acquisition (DDA) mode, using a Top10 method. MS1 resolution was set to 70,000 (at 200 *m/z*), mass range of 375–1650 m/z, AGC of 3×10^6 and maximum injection time was set to 60 ms. MS2 resolution was set to 17,500, quadrupole isolation 1.7 *m/z*, AGC of 1×10^5, dynamic exclusion of 45 s and maximum injection time of 60 ms. Only charge states from 2 to 8 were allowed for MS/MS triggering.

2.5. Database search and peptide analysis

Peptides were searched against a database containing available nematode protein sequences in UniProtKB (February 2019, http://www.uniprot.org/) using the PEAKS Studio 7.5 Software (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) and verified with the Protein-protein Basic Local Alignment Search Tool (BLASTp) accessed at the National Centre for Biotechnology Information (NCBI) website (February 2019, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PEAKS 7.5 software used the *de novo* peptide sequencing results for a standard database search with 125 common laboratory contaminants and worms' proteins from the Nematoda Chromadorea containing 1,233,685 entries from all available protein sequences from the members of this group, with a false discovery rate (FDR) of < 1%. Search parameters included a parent mass error tolerance of 10.0 ppm, fragment mass error tolerance of 0.02 Da, methionine oxidation and asparagine deamidation as variable modifications, cysteine carbamidomethylation as fixed modification and maximum 1 missed trypsin cleavage. In addition, an open modification/variant database search with the Spider algorithm was performed for the search of potential mutations in *S. lupi* sequences. The total ion current (TIC) normalized intensities of the annotated features were used as the quantitative index for comparisons and reported as the label free quantification (LFQ).

For the BLASTp analysis, the peptides obtained from the PEAKS analysis were searched for protein homologues of the Nematoda group database (taxonomy id: 6231) which contains 1,550,191 protein sequences. The search parameters included filtering of the results with a Max score higher than 30 and an expected value (*E*-value) lower than $1e^{-4}$. Finally, identity percentages obtained in the BLASTp search were registered for each peptide. Peptides with the same protein annotation in both databases were considered to belong to the same parent protein.

The exponentially modified Protein Abundance Index (emPAI) and the coverage percentage values were calculated for each identified protein in each developmental stage. First, the PeptideMass program (Wilkins et al., 1997), available at the ExPASY server (https://web.expasy.org/peptide_mass/), was used to predict the number of theoretical tryptic peptides in each protein, allowing no missed cleavages. Then, the obtained peptides belonging to the same parent protein were counted and compared to the theoretical number of tryptic peptides, and the emPAI was calculated as previously described (Ishihama et al., 2005). The coverage of every protein found in each developmental stage was estimated by the sum of the length of all identified peptides and divided by the theoretical total length of the protein homologue.

Finally, a Venn diagram was drawn using only proteins with an appended description to show all possible expression relations in the different life stages. For this purpose, the VennDIS 1.0.1 tool (Ignatchenko et al., 2015) was employed.

2.6. Functional annotation

Gene Ontology (GO) analysis was done with the QuickGO (https://www.ebi.ac.uk/QuickGO/) and the WormBase (www.wormbase.org) websites. The biological process, molecular function and cellular compartment hierarchical categories available for the specific proteins were obtained from the WormBase website. Statistical tests were performed to estimate the difference in the protein associated GO terms in each the developmental stages with a Fisher's two tailed exact test after Bonferroni correction for multiple comparisons using the WinPepi

11.43 software (Abramson, 2011). Then, a gene ontology enrichment analysis of the biological process and cellular compartment annotations was done for those proteins detected in all stages or uniquely found in a particular developmental stage with the PANTHER 14.0 Overrepresentation Test. For this aim, the *C. elegans* database was used as a reference and the Fisher's exact test with Bonferroni correction for multiple testing was employed (Thomas et al., 2003, 2006). Since the genome of *S. lupi* or other parasitic nematode was not among the supported reference proteome genomes, a PANTHER Generic Mapping file list was generated to match the peptides of *S. lupi* obtained with the PANTHER hidden Markov models (HMM) library v14.1. Statistically significant overrepresented GO terms were graphically summarized using the REVIGO online tool (Supek et al., 2011). The semantic similarity of the GO terms was estimated through SimRel (allowed similarity = 0.7). Then, the semantic space versus the $\log_{10}$ (*p* value) were plotted with the "ggplots2" (Ito and Murphy, 2013), "scales" and "ggrepel" packages in R (Team, 2013).

2.7. Quantitative analysis

This analysis was performed using the Perseus v1.6.0.7 software (Tyanova et al., 2016). Intensity values obtained from the LFQ were $\log_2(x)$ transformed. After this, proteins detected in only one biological replicate were filtered out. Missing values were replaced by the constant zero. Then, a principal component analysis (PCA) graph was constructed to determine the correlation in the protein composition in each replicate for all developmental stages studied. Scatterplots from the replicates of each life stage were drawn in order to calculate the Pearson correlation coefficients. A heatmap was plotted with the $\log_2(x)$ transformed intensity values normalized by the Z-score calculation only from those proteins detected in all biological replicates from the four analyzed developmental stages. The heatmap used the Pearson correlation and the Euclidean clustering method for columns and rows, respectively. Fold changes in the $\log_2(x)$ transformed protein intensities were calculated between stages, in which +1 or -1 values indicate doubling or reduction in 50% in the detected intensities, respectively.

3. Results

3.1. Identification of proteins in the L3, L4 and adult stages of S. lupi

A total of 838 peptides were obtained from the *S. lupi* excretion-secretion proteins of all developmental stages, according to the peptide search procedure explained above (Supplementary Table. S1).These corresponded to 211 proteins, 171 with an appended description and 40 uncharacterized (Supplementary Table. S2). These 211 proteins were most similar to 38 different nematode species, mainly vertebrate parasites but also to some plant parasitic and free-living nematodes. L3 larvae, L4 females, adult females and adult males expressed 117, 130, 99 and 116 proteins, respectively. Most of these proteins shared the highest similarity to *Toxocara canis* (10.4%, 22/211), *Onchocerca flexuosa* (9.5%, 20/211) and *Pristionchus pacificus* (9.0%, 19/211). Moreover, 72% (152/211) of the proteins were 100% similar to a reference database, whereas 19.0% (40/211) were 90–99% similar and 9.0% (18/211) were 60–89% similar to these references. Eighteen percent (27/152) of the proteins with 100% similarity to the databases were expressed by all developmental stages, while the rest were identified in a differential pattern according to the stage. In addition, those with the lowest similarity were mainly expressed by L4 females (15/18).

The molecular weight (MW) of the proteins from the parasitic homologues ranged from 8.97 to 865.68 kDa, with most of them being in the range of 9.29 to 59.29 kDa (64.0%, 135/211) and 59.30–109.29 (22.3%, 47/211) (Supplementary Table. S2). The smallest proteins identified were a disulfide isomerase precursor (MW: 9.29 kDa) homologous to *Ancylostoma caninum* and the dynein light chain 1 (MW: 9.32 kDa) homologous to *Onchocerca volvulus*. The largest detected proteins were expressed by L4 females only and were homologous to the calcium binding EGF domain-containing protein of *O. volvulus* (MW: 350.84 kDa), the transmembrane cell adhesion receptor mua-3 of *T. canis* (MW: 400.11 kDa) and an uncharacterized protein FL81_12969 from *Caenorhabditis remanei* (MW: 865.68 kDa).

The coverage and emPAI values differed among the proteins identified and the life stages expressing them (Supplementary Table. S2). The coverage percentages ranged from 0.17% to 79.52% corresponding to the uncharacterized protein FL81_12969 and the cyclophilin-type peptidyl prolyl cis-trans isomerase. This last protein was expressed in all developmental stages, and its coverage in the four studied life stages ranged from 45.7% to 79.5% in L3 larvae and L4 females, respectively. The emPAI values varied in the similar direction to the coverage percentages, since they represent the overall abundance of the protein in the sample and can theoretically range from 0 to 9, corresponding to the lowest and the highest abundance, respectively. The proteins with the smallest emPAI values were the spectrin beta and alpha chains, the transcription factor 3C subunit, the uncharacterized protein FL81_12969 and the uncharacterized protein PRIPAC_35208, all with emPAI values of 0.01. Finally, the highest emPAI of 5.42 was obtained with the tubulin beta chain detected in L3 larvae.

Variable combinations of protein expression by the different *S. lupi* developmental stages were obtained (Fig. 2; Supplementary Table. S3). In total, 25.7% (44/171) of the proteins with appended description were identified in all developmental stages, 36.8% (63/171) were shared in two to three life developmental stages, whereas 37.4% (64/171) were unique to a particular life developmental stage, according to the described *in vitro* conditions. The glyceraldehyde 3-phosphate dehydrogenase (Han et al., 2012), fatty acid retinoid binding protein (Zhan et al., 2018) and galectin (Yanming et al., 2007) were among the proteins detected in all life stages which may be of particular further interest. This category also includes the major sperm protein (Gozdzik et al., 2012; Zottler et al., 2017) detected at a higher intensity in male adults compared to female adults, and not identified at all in the L3 and L4 stages. Notably, L4 females expressed the largest number of unique proteins (28.6%, 49/171), including cuticlin-1, basement membrane proteoglycan, transmembrane cell adhesion receptor mua-3 and the Ov16 antigen, which has been used as a diagnostic antigen for *O. volvulus* (Lagatie et al., 2018).

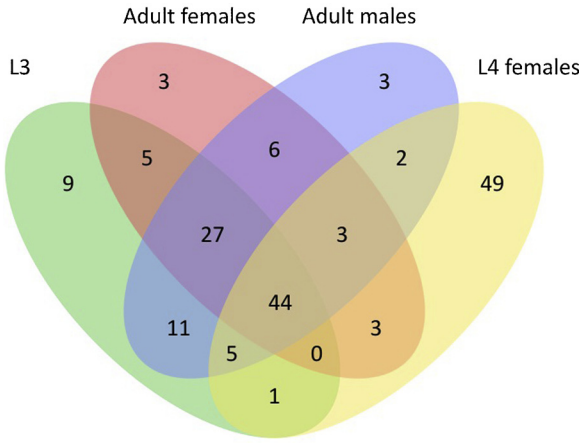

Fig. 2. Venn diagram of the proteins detected in different *S. lupi* developmental stages. Common and unique proteins detected in L3 larvae (green oval), adult females (red oval), adult males (blue oval) and L4 females (yellow oval) are illustrated. The analyzed proteins correspond to those with an appended description in the databases.

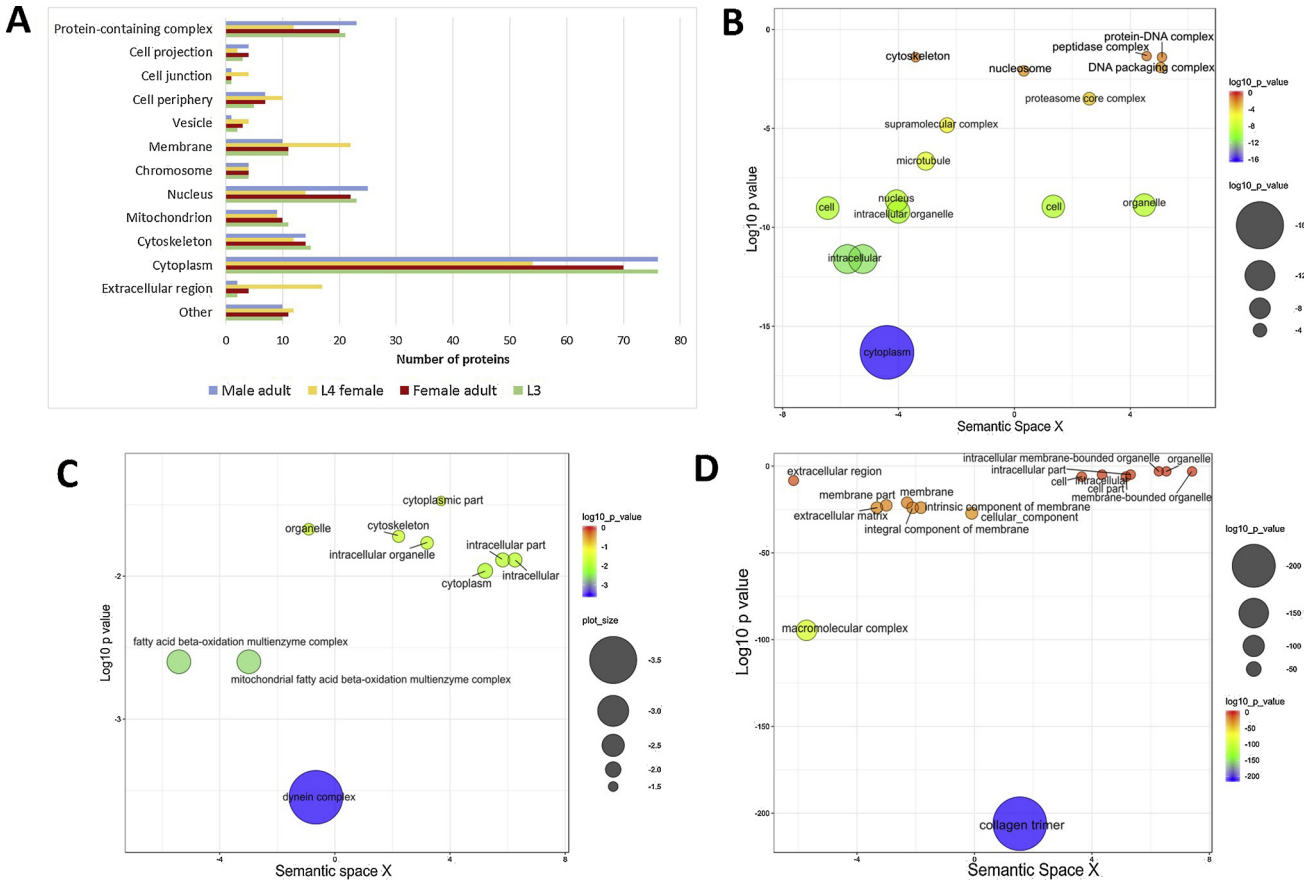

Fig. 3. Gene ontology (GO) terms relating the cell compartment assigned to the proteins identified in the secretions of different *S. lupi* developmental stages. The summarized significant cell compartment classification of proteins shared by the four life stages analyzed (A, B), and the unique proteins found in L3 larvae (C), and L4 females (D), are shown as scatterplot views from the REVIGO category clusters. Color and size of the circles are proportional to the $\log_{10}$(p-value), in which blue and larger circles represent more significant values, according to the scales of each graph. The organization of semantically similar GO terms is illustrated in the x-axis.

3.2. Functional analysis

The Gene Ontology (GO) analysis revealed that *S. lupi* PES were associated with a variety of cellular compartments and biological processes. GO annotations were available for 87.7% (150/171) of the total proteins with an appended description. Those that were not functionally annotated (12.3%, 21/171) were found mostly in L4 females.

The proteins related with cellular compartments and detected in all *S. lupi* stages had GO terms mainly associated to the cytoplasm (Fig. 3A and Supplementary Table. S4). Accordingly, 65% (76/117), 45.5% (54/130), 70.7% (70/99) and 65.5% (76/116) of the identified proteins of L3, L4 females, adult females and adult males, respectively, had a cytoplasmic localization, such as the fatty-acid retinoid binding protein and the aspartate aminotransferase. Moreover, 19.5% (23/117), 10.7% (14/130), 22.2% (22/99) and 21.5% (25/116) of the proteins of L3 larvae, L4 females, adult females and adult males, respectively, were associated to the nucleus, such as the transcription factor three C subunit or histones H2A or H4. Additionally, the less represented GO cellular compartments were cell projection (i.e. four proteins), cell junction (i.e. five proteins) and vesicle-associated (i.e. seven proteins). Remarkably, the L4 females significantly expressed more proteins (13.1%, 17/130) associated to the extracellular region than the other developmental stages (Fisher's exact test, two tailed, all $p < 0.008$), including alpha amylase, A2-macroglubulin, basement membrane proteoglycan, collagen protein 109, cuticlin-1, pepsin inhibitor, and the transmembrane cell adhesion receptor mua-3. In addition, the only molecules from the extracellular compartment that were shared by all stages were the galectin and transthyretin-like proteins. Interestingly, L4 females also expressed a higher number of proteins associated with membranes in comparison to the L3 and adults, however, these differences were marginally non-significant (Fisher's exact test, two tailed, all $p < 0.083$).

The enrichment analysis for the cell compartment annotations estimated 18 statistically significant localizations, for the proteins shared by all life stages, when compared to the *C. elegans* database (Table 1; Supplementary Table. S5). This data confirmed the results above by showing an overrepresentation of cytoplasmic localization of *S. lupi* proteins (all $p < 9.x10^{-6}$) (Fig. 3B). Regarding unique proteins associated with a particular life stage, 14 cell compartment annotations were significantly enriched in the L3, which were most significantly located in the dynein complex and cytoskeleton (all $p < 0.005$) (Fig. 3C). In L4 females, 21 cell annotations were enriched including the collagen trimers, extracellular matrix and protein complexes which were the most significantly enriched (all $p < 0.00001$) (Fig. 3D). In contrast, adult females and adult males did not show any statistically significant cell compartment enrichment (Table 1).

The most abundant GO annotations for biological processes shared in all stages were small molecule metabolism and regulation of processes (Fig. 4A and Supplementary Table. S6). For instance, 21.5–30.8% of the proteins identified in all life stages were associated with small molecule metabolism, such as catalase or glutamate dehydrogenase. In addition, 21.5–32.3% of the proteins were involved in the regulation of processes, including the Ras family protein and tropomyosin. In contrast, DNA metabolic process annotation was among the least represented biological process, with only five detected proteins in all stages (i.e. 26S proteasome non-ATPase regulatory subunit 14, 40S

Table 1
Gene Ontology Enrichment analysis. The analysis was performed for the uniquely expressed proteins from each *S. lupi* developmental stage and the proteins identified in all stages. The analysis was done by using the *Caenorhabditis elegans* GO biological process and cell compartment databases.

Set of proteins	Mapped IDs to the reference database	Number of cell compartment enriched annotations*	Number of biological process enriched annotations**
Shared by all stages	20	25	42
Unique in L3 larvae	4	14	29
Unique in L4 females	23	21	62
Unique in adult females	2	0	0
Unique in adult males	0	0	0

* Statistically significant after the Bonferroni correction with 697 independent tests.
** Statistically significant after the Bonferroni correction of 2414 independent tests.

ribosomal protein, FACT complex, polyubiquitin-like protein and purine nucleoside phosphorylase), and was followed by cell proliferation with only one protein identified in L4 females (the cofilin/tropomyosin-type actin binding protein). Importantly, the GO category "Other" which includes specific processes not contained in the other annotations was the most represented.

Functional enrichment in GO categories of biological processes was found in 42, 29 and 47 subcategories in the proteins shared by all developmental stages, L3 unique proteins and L4 female unique proteins, respectively (Table 1 and Supplementary Table. S7), when compared to the *C. elegans* database. Interestingly, functional enrichment of protein dephosphorylation, phosphorus metabolic processes and organic nitrogen compound metabolic processes were overrepresented among the proteins detected in all life stages ($p < 2.1 \times 10^{-9}$) (Fig. 4B). Moreover, fatty acid oxidation, lipid metabolism pathways, and monocarboxylic acid metabolic processes were significantly enriched ($p < 0.00005$) among the L3's unique proteins (Fig. 4C). With regard to the L4 females' unique proteins, the three most enriched biological processes were the mannose metabolism, protein de-glycosylation and carbohydrate metabolism ($p < 0.00004$) (Fig. 4D). Moreover, several additional processes were also significantly enriched in L4 females, such as the cellular response to stimuli, N-glycan processing and cell-junction assembly and organization ($p < 0.0009$).

3.3. Quantification analysis

Principal component analysis (PCA) showed a high similarity in the protein composition of the biological replicates of each *S. lupi* developmental stage. The first, second and third components of the PCA explained 61.3%, 17.2% and 10.8% of the associations between samples, respectively (Fig. 5A and 5B). Adult females and males were highly similar in the PCA's first and second components (Fig. 5A), however, the protein composition of L3 larvae and L4 females was distant from that of the adult stages. In addition, a strong positive

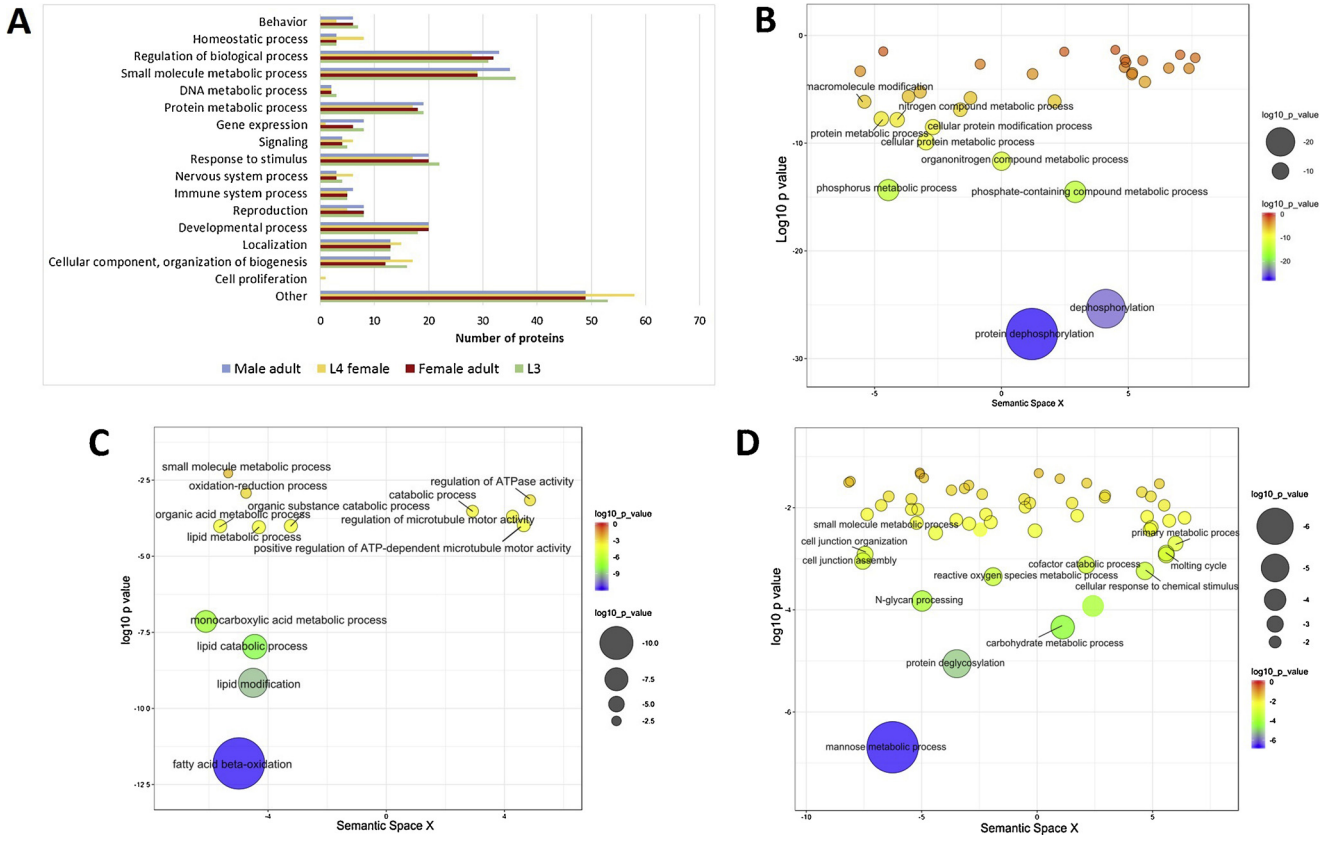

Fig. 4. Gene ontology terms of the biological process assigned to the proteins identified in the secretions of *S. lupi* different developmental stages. The summarized significant biological process classification of the proteins shared by the four life stages analyzed (A, B), and the unique proteins found in L3 larvae (C), and L4 females (D), is shown as scatterplot views from the REVIGO category clusters. Color and size of the circles are proportional to the $\log_{10}$(p-value), in which blue and larger circles represent more significant values, according to the scales of each graph. The organization of semantically similar GO terms is illustrated in the x-axis.

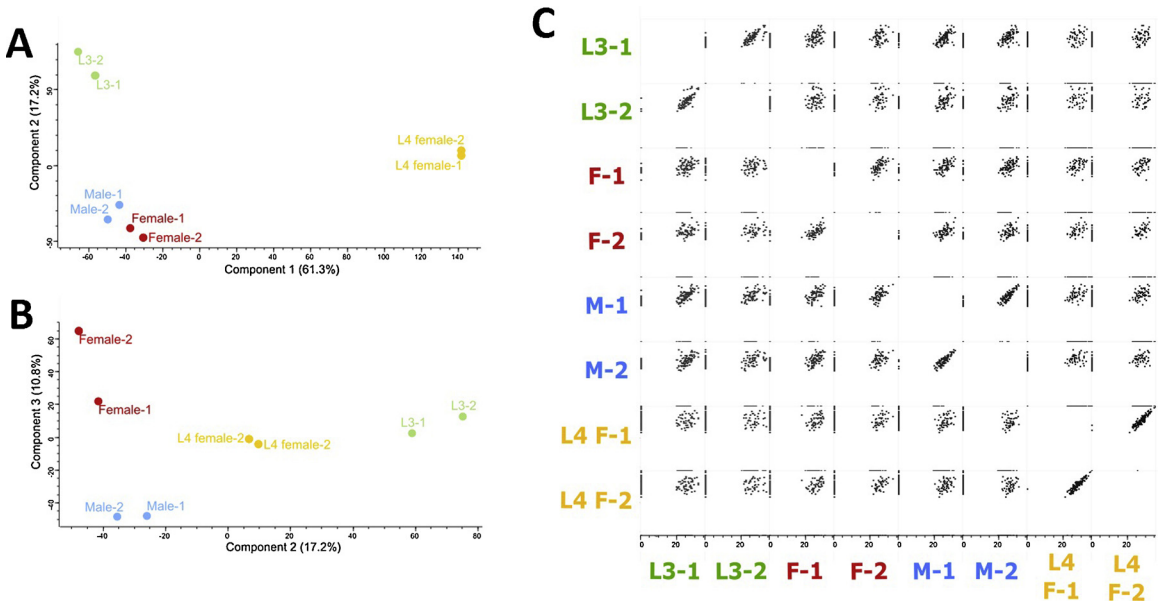

Fig. 5. Correlation of the protein composition in the biological replicates from each *S. lupi* developmental stage. (A, B) Principal component analysis scatter plots show the first, second and third components in the correlation of the protein composition from each biological replicate. (C) The multi-scatterplot compares the protein composition between all biological replicates.

correlation between biological replicates was shown by the scatterplots (Fig. 5C), with Pearson correlation coefficients for L3s, L4 females, adult females and adult males' biological replicates of 0.837, 0.943, 0.730 and 0.851, respectively.

The detection of some proteins was two-fold higher in certain developmental stages compared to others, as found with fold changes (FC) values higher than one in the $\log_2(x)$ transformed intensities (LTI) (Fig. 6 and Supplementary Table. S8). These proteins include: calmodulin (FC = 1.14–1.23), heat shock protein 70 (FC = 1.15–1.31), histone H2B (FC = 1.06–1.24), tubulin alpha (FC = 1.24–1.44) and beta chains (FC = 1.12–1.24), which were detected more frequently in L3 larvae than in the other stages. In addition, the 14-3-3 protein (FC = 1.07–1.20), cyclophilin-type peptidyl-prolyl cis-trans isomerase (FC = 1.09–1.22), cytosolic non-specific dipeptidase (FC = 1.13–1.48), moesin/ezrin/radixin homolog 1 (FC = 1.16–1.37), the Ras family protein (FC = 1.20–1.48) and transthyretin/like protein (FC = 1.04–1.15) were expressed more in L4 females than the other life stages. The enolase (FC = 1.01–1.20) and pyruvate kinase (FC = 1.04–1.24) enzymes were expressed two times higher in male adults; and, the protein disulfide isomerase (FC = 1.01–1.29) was detected at a larger proportion in females compared to other stages. Moreover, the fatty acid retinoid binding protein and glutamate dehydrogenase were equally detected in adult females and males (FC = 1.00), and higher than in L3 or L4 females. Interestingly, the abundance of the annexin 6 protein was similar in L4 females (LTI: 22.64, 21.82), adult females (LTI: 19.72, 20.47) and adult males (LTI: 19.70, 18.54), and higher in these three stages than in L3 larvae (16.04, 14.98).

4. Discussion

Helminths have adapted to a myriad of niches within a variety of different of hosts, ranging from arthropods to vertebrates. They communicate and undertake their penetration, migration and establishment within these hosts through active secretion or passive release of proteins into the environment in which they develop (Sotillo et al., 2017). *S. lupi* makes a good model for the study of these molecules and interactions, as it makes a long journey from the infective larval stage (i.e. L3), found in the worm's intermediate host (i.e. dung beetle), to the development of adult specimens in the definitive host (i.e. dog). The pathway of this journey is associated with a variety of pathological processes in the definitive host, such as the formation of aortic aneurisms, esophageal nodules and fibro- and osteosarcomas (van der Merwe et al., 2008). In addition, molecules involved in excretion-secretion have been useful for the design of vaccines and diagnostic assays (Anderson et al., 2015; Chen et al., 2011; Gozdzik et al., 2012; Mehrdana and Buchmann, 2017). In the present study we demonstrated that different developmental stages of this parasite secrete a wide range of proteins, including shared and life stage-specific molecules. In addition, this study sheds light on the helminth-host interactions and suggests how each developmental stage interacts differently with it host (i.e. L3 larvae versus L4 and adult stages) and how this can change according to the localization within the host (i.e. hemocoel inside the beetle versus aorta and esophagus in the dog).

A total of 211 proteins were detected in all *S. lupi* stages, 81% of these with an appended annotation, and 19% identified as uncharacterized or hypothetical according to the UniProtKB and NCBI databases. Interestingly, a larger amount of proteins was identified from L4 female secretions (n = 130) than from L3 larvae (n = 117), adult males (n = 116) or adult females (n = 99), with a high correlation in the protein composition between the biological replicates from each developmental stage (all Pearson correlation > 0.730). A previous study detected only nine proteins in the media of *S. lupi* adults and their maintenance in the laboratory was limited to 6 days (Sako et al., 2017). Herein, the use of nano-UPLC MS/MS *de novo* peptide sequencing enabled us to detect and successfully characterize a much higher number of proteins. Moreover, the culture conditions described in the present study demonstrated higher tolerance of the worms than those described by Sako et al. (Sako et al., 2017), since adult males and females in the current study were maintained viable and free of bacterial and fungal contamination for 16 and 36 days, respectively.

The predicted secretomes of Clade III nematodes in which *S. lupi* is included, i.e. the theoretical list of secreted proteins of an organism based on classical and non-classical signal sequences in the expressed sequence tags, ESTs, or genomes (Garg and Ranganathan, 2012), usually comprise a larger set of proteins than the number of proteins detected herein. For instance, 517 secreted proteins were predicted in the human lymphatic filarioid *Wuchereria bancrofti,* 593 in the dog heartworm *Dirofilaria immitis* and 858 in the dog ascarioid *Toxocara canis* (Cuesta-Astroz et al., 2017). However, the composition of the

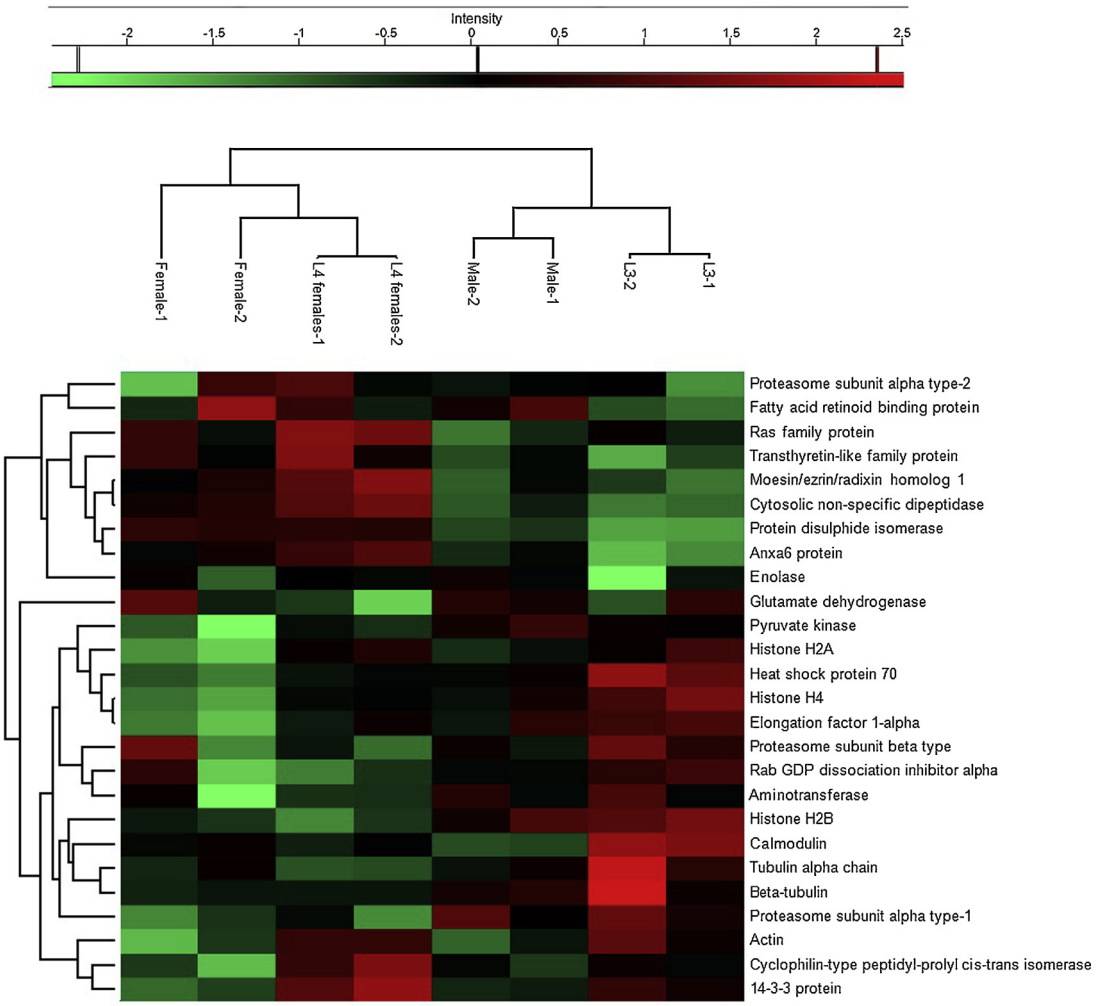

Fig. 6. Heatmap of the proteins detected in all *S. lupi* biological replicates. Their annotations are shown on the right. Transformed $\log_2(x)$ intensity values normalized with the Z-score were used to color-code the protein intensities.

expressed secretome described in *T. canis*-larvae has ranged from 19 (Sperotto et al., 2017) to 64 proteins (da Silva et al., 2018) in different studies using similar analytical approaches to those employed in the present study. This highlights that predicted secretomes are usually composed of a larger set of proteins than the expressed secretomes. In accordance with this, the predicted secretome of the lymphatic filarioid *Brugia malayi* consists of 800 proteins (Cuesta-Astroz et al., 2017), but its expressed secretome changes according to the life stage, i.e. 160, 119 and 76 proteins in adult females, adult males and L3, respectively, with only 32 shared by all stages (Moreno and Geary, 2008). In our study, a differential pattern of protein expression according to each studied stage was found. Forty-four of the proteins were shared among all developmental stages and 64 were exclusively detected in a particular stage. Of these, 49 belonged to L4 females. These previous studies confirmed that each life cycle stage of a given parasitic helminth secretes hundreds of proteins, often with little conservation between the developmental stages (Sotillo et al., 2017). This variety of proteins might predict the diverse worm's metabolic needs, its interaction with the host or to some extent with its *in vitro* culture conditions (Hewitson et al., 2009). In complete agreement with the latter, our data showed how dynamic the *S. lupi* expressed secretome is and how it adjusts to the developmental stage and the conditions to which each stage is exposed.

The secretome of *S. lupi* was found homologous to other nematode proteins, as expected due to the comparison to available nematode databases. Accordingly, 85% of the obtained peptides were 100% identical to other nematode protein sequences available, and just 15% of the identified peptides exhibited only 60–99% identity to the used databases. Once the *S. lupi* genome and transcriptome databases are available, proteins with lower percentage of homology will be properly identified. The detected peptides showed the highest homology to proteins of *T. canis* and the deer filarioid *Onchocerca flexuosa*. This high similarity can be explained by sharing the same canid host with *T. canis*, or in the case of *S. lupi* and *O. flexuosa*, by their close phylogenetic relatedness (Rojas et al., 2018). Moreover, some protein domains conserved among all nematode predicted secretomes were identified in our data, namely, the thioredoxin and immunoglobulin I-set domains, involved in redox signaling and cell adhesion, respectively (Cuesta-Astroz et al., 2017). However, other domains and proteins commonly detected in other nematodes, such as the Kunitz/Bovine pancreatic trypsin inhibitor domain, the Homeobox domain and the Cystein-rich secretory proteins, antigen 5, and pathogenesis-related 1 domain-corresponding proteins, were not detected in our database. This might be explained by lack of expression in the conditions described herein.

The proteins detected in the four analyzed life stages were significantly enriched in phosphorus metabolism, including protein dephosphorylation, and metabolic processes of phosphate-containing proteins, when compared to the *C. elegans* database. Accordingly, the serine/threonine phosphatases (PP), triosephosphate isomerase, pyruvate kinase and glucose-6-phosphate isomerase, were detected in all *S. lupi* samples. These proteins have cytoplasmic localization, which was the most enriched cellular compartment from the proteins shared by all stages. PPs are key regulators in developmental and reproductive

processes of other helminths. For example, PP1 has been identified in the reproductive tissues of the human fluke *Schistosoma japonicum*, and seems to mediate the maturation of reproductive organs (Zhao et al., 2018). In addition, *T. canis*-associated PP has been detected in adult males and assumes an important role in spermatogenesis (Ma et al., 2014, 2015). Furthermore, the enzymes triosephosphate isomerase, pyruvate kinase and glucose-6-phosphate isomerase are required for pyruvate metabolism and ATP and carboxylic acid biosynthesis which ultimately reflect the role of carbohydrates as a main energy source, as observed for *Haemonchus contortus* (Kapur and Sood, 1987). Overall, the enrichment of these pathways in *S. lupi,* implies high energy demand in the different life stages and potential use of phosphatases for maturation and reproduction.

S. lupi-associated PES might play a role in development of fibro- or osteosarcomas (Dvir et al., 2010; Meléndez and Suárez-Pellín, 2001), as shown for the oncogenic trematode *Opistorchis viverrini* (Smout et al., 2009). In this study, the only potential candidate found for a carcinogenic process was annexin 6. This protein was detected in all life cycle stages, but, at a larger proportion in those that develop in the dog host. Annexin 6 is a member of a conserved superfamily of calcium-dependent membrane binding proteins and has several functions including cell adhesion and signal transduction (Qi et al., 2015), and has been of increasing interest due to their role in host-pathogen interactions in platyhelminthes (Cantacessi et al., 2013). Its orthologue in humans has been associated with different types of cancer and osteoarthritis. For instance, a high expression of annexin 6 has been correlated with cervical cancer (Lomnytska et al., 2011), lymphoblastic leukemia (Smith et al., 2002) and pancreatic adenocarcinoma (Leca et al., 2016). Conversely, down-regulation of annexin 6 was associated with melanoma (Francia et al., 1996), breast cancer (Sakwe et al., 2011) and gastric cancer (Wang et al., 2013). Despite the fact that annexin 6 has many other roles in normal cell homeostasis and that annexins in helminths and vertebrates have diverged and serve different functions, it is a prospective candidate for the carcinogenic development in spirocercosis. Thus, the functional analysis and the potential role of this molecule in neoplasia induced by *S. lupi* requires further exploration and confirmation.

Functional enrichment of fatty acid and lipid metabolism was observed in L3 larvae-unique proteins detected in the PES, when compared to the *C. elegans* database. Namely, the enoyl CoA hydratase and acyl CoA dehydrogenase, which are essential for the beta-oxidation of fatty acids were enriched in the secretome of L3 larvae. The significant overrepresentation of such a process can be explained by: i) adaptation to the beetle's internal high fatty acid and lipid content (Barroso et al., 2014), ii) the change in metabolism of the worm's cuticle lipid composition after infection of the canid host, as demonstrated for *Acanthocheilonema viteae* and *Ostertagia ostertagi* when mimicking the internal conditions of their definitive hosts (Proudfoot et al., 1990), or iii) increased fatty-acid metabolism as energy reservoir in L3 larvae, as suggested for the non-feeding L1 to L3 stages of *Dictyocaulus viviparus* (Becker et al., 2017). Therefore, it can be hypothesized that fatty-acid oxidation might also be associated with energy production in the infective stages of *S. lupi*.

S. lupi L4 stages migrate through the dog's aorta to finally reach the esophagus (Sen and Anantaraman, 1971). Concomitantly, these stages change their cuticle, produce sexual structures and increase their size to finally develop into adult females and males (Sen and Anantaraman, 1971). This transforming phenotype was corroborated with the functional and compartment enrichment analyses of the secreted proteins detected only in L4 females. Accordingly, the alpha-mannosidase, a key enzyme for the synthesis of glycosylated proteins, abundant in the extracellular matrix (ECM) (Wilson et al., 2015), was functionally enriched in these larvae. Furthermore, proteins belonging to the ECM were enriched in L4s, i.e. cuticlin-1, basement membrane proteoglycan (also known as perlecan) and the protein qua-1, which have an active role in molting and muscle development (Hao et al., 2006; Mullen et al., 1999). In addition, other L4-detected proteins such as cathepsin B-like cysteine proteinase 6, cathepsin S-like cysteine proteinase, alpha-*N*-acetylgalactosaminidase, collagen 109, contactin-associated protein-like 5, fibronectin type III domain protein and G2F domain protein, are basic components of the ECM or interact with it (Riddle et al., 1997). Remarkably, the transmembrane cell receptor mua-3 was uniquely detected in L4 females with a high coverage. This large protein contains an epidermal growth-factor, von Willebrand type A, low-density lipoprotein and sea urchin-enterokinase-agrin domains (Bercher et al., 2001). The transmembrane cell receptor mua-3 together with the protein let-805, also detected in L4 females, are required for hypodermal-cuticle attachment during the worm's growth as found in *C. elegans* (Hetherington et al., 2011). Therefore, the differential protein expression detected in *S. lupi* L4 females reflects the concurrent molting process to adult stages and the interaction with the ECM derived from the worm itself or the host environment, necessary for their migration.

The cytoplasmic compartment was enriched in the PES shared by all *S. lupi* life stages. One possible explanation for this finding is the release of cytoplasmic proteins as a consequence of cell turnover or the shed of these molecules contained in extracellular vesicles (EVs), as has been found for *D. immitis* (Tritten et al., 2016) and *F. hepatica* (de la Torre-Escudero et al., 2019). Future studies on *S. lupi* EVs will disclose the identity of this parasitic cargo, which may include proteins, as well as RNA and DNA responsible for host-pathogen interactions (Eichenberger et al., 2018).

A variety of immune regulating proteins detected in other nematodes, were also identified in all *S. lupi* developmental stages. For example, the fatty acid and retinol-binding family proteins have been shown to inhibit the host's lipid mediated immune responses in *Onchocerca volvulus* (Bradley et al., 2001), the thioredoxin peroxidase counteracts the action of reactive oxygen species released by innate immune cells in *O. volvulus* (Lu et al., 1998) and induces the alternative activation of macrophages in *Fasciola hepatica* infection (Donnelly et al., 2005). Moreover, galectin, detected with high coverage in our study in all studied developmental stages, has been linked to the parasite's invasion or the regulation of the host immune system towards a Treg lymphocyte response to *Toxascaris leonina* (Kim et al., 2010). Furthermore, a-2-macroglobulins detected in secretions from L4 and adult female *S. lupi* may be involved in reducing blood clotting at feeding sites as observed for tapeworms (International Helminth Genomes, 2019) and additionally, it may modulate the host immune response, through binding to several cytokines and hormones (Rehman et al., 2013). The transthyretin-like family protein and cyclophilin-type peptidyl-prolyl cis-trans isomerase-15 have been both implicated in the induction of Treg responses in *Schistosoma mansoni* (Floudas et al., 2017; Hewitson et al., 2009). Hence, the detection of homologues of such proteins in *S. lupi* secretions suggests that this worm leads to a Treg immune response, potentially associated with chronic infection of its definitive hosts. However, histochemical analysis of the infiltrating immune cells in benign and neoplastic nodules induced by *S. lupi*, have indicated a predominating pro-inflammatory response with no detected Treg cells (Dvir et al., 2011). Therefore, the role of these secreted proteins must be ascertained in order to clarify the course of the immune response during *S. lupi* infection. Furthermore, the detection of secreted immunoregulatory molecules in the L3 stages suggests that perhaps the same protein may have different roles in the definitive and intermediate hosts, since these two hosts differ in their responses against parasites.

Some of the proteins detected in *S. lupi´s* PES have been used for the diagnosis of infection with other helminths and may also be useful for this purpose in *S. lupi*. The major sperm protein detected mainly in *S. lupi* adult males, has been used as a diagnostic marker for the cattle and cat lungworms *Dictyocaulus viviparus* (Gozdzik et al., 2012) and *Aelurostrongylus abstrusus* (Zottler et al., 2017). In addition, the Ov16 protein, detected in L4 females of *S. lupi,* has been used as antigen for the detection of IgG4 in *O. volvulus*-infected human patients (Lagatie et al., 2018; Wilson et al., 2016). In this regard, only one serological test using

crude antigens of an adult *S. lupi* male has been developed but not currently used for diagnosis (Coskun, 1995). Thus, the PES described herein have potential for the development of immunological-based diagnostic assays.

Helminth-associated PES identified in this study in *S. lupi* have also shown to be highly immunogenic and used as vaccine antigens for other helminths. The fatty acid-retinoid binding protein of *B. malayi* (Zhan et al., 2018), glyceraldehyde 3-phosphate dehydrogenase of *H. contortus* (Han et al., 2012) and *O. volvulus* (Steisslinger et al., 2015), galectin of *H. contortus* (Han et al., 2012; Yanming et al., 2007) and cathepsin B of *Trichinella spiralis* (Yang et al., 2018) have shown to reduce the worm burden in different animal models and protect against subsequent infections. In addition, vaccination using complete ES products has been attempted in mice infected with *Trichiuris muris* (Shears et al., 2018), and shown to stimulate long lasting protection against infection with this parasite. Moreover, metallopeptidases expressed in *Schistosoma mansoni* (Dvorak et al., 2016) and enzymes secreted by *C. elegans* during collagen formation and identified in other parasitic nematodes (Page et al., 2014) and in *S. lupi*, have been analyzed for their potential as drug targets. Thus, the immunogenic potential of the molecules mentioned above and other proteins detected herein, may be evaluated as potential vaccine and drug candidates against this potentially fatal disease of dogs.

The analysis of the PES in some other worm species has been facilitated with the genome and ESTs sequencing (da Silva et al., 2018; Sperotto et al., 2017). Thus, the sequencing of the genome and transcriptomes of *S. lupi* might lead to uncovering of new proteins involved in the pathogenesis of the disease, not detected in the present study, or detected with a low homology, or with more than one post-translational modification (Jex et al., 2019). In addition, a proteogenomics approach, which combines proteomic data with genome sequencing, can increase the annotation of the worm's proteins and genes, ultimately leading to the discovery of novel interactions (Sotillo et al., 2017). However, this was beyond the scope of current study.

S. lupi establishes complex interactions with its hosts by the secretion of an arsenal of proteins expressed in different patterns according to each life stage. Proteins shared by the different stages are mainly involved in energy metabolism, development and maturation, whereas unique proteins expressed in L3 larvae and L4 females are implicated in fatty-acid oxidation and extracellular matrix interactions, respectively. Interestingly, the protein annexin 6 was expressed more abundantly in L4 and adult *S. lupi* stages than in L3 larvae and stands as a potential candidate to study the mechanisms leading to neoplasia. Overall, the proteins identified in this study present a promising potential for use as diagnostic, drug or vaccine targets.

Credit author statement

AR and GB conceptualized the study, analyzed the data and wrote the first draft of the manuscript. AR performed all laboratory experiments. GB acquired the funding and supervised the project. Both authors reviewed and edited the paper.

Declaration of Competing Interest

The authors declare there is no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2019.108935.

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