**The somatic proteins of *Toxocara canis* larvae and excretory-secretory products revealed by proteomics**

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**Abstract:** Toxocariasis is a widespread helminth infection of dogs and cats, caused by *Toxocara canis* and *Toxocara cati* larvae, respectively. *Toxocara* spp. can cause zoonotic infections in humans by invading tissues and organs causing pathology. *Toxocara* spp. larvae release excretory-secretory molecules (TES) into the body of their host that are fundamental to the host-parasite interaction and could be used as targets for novel diagnostics and vaccines. In the present study, we identified 646 *T. canis* proteins from TES and larval extract using 1D-SDS PAGE followed by mass spectrometry. A wide range of proteins was identified that may play a role both in the induction of the host immune response and host pathology, and in parasite metabolism and survival. Among these proteins there are potential candidates for novel diagnostics and vaccines for use in humans and natural animal hosts.

Keywords**:** *Toxocara canis*; Toxocariasis; Proteomics; Excretory/secretory proteins; Proteomics.

**1. Introduction**

The nematodes *Toxocara canis* and *Toxocara cati,* whose definitive hosts are dogs and cats, respectively, are the causative agents of human toxocariasis, a neglected zoonotic infection with a worldwide distribution. Toxocariasis is considered to be of significant public health relevance, particularly in low and middle-income countries among populations living in conditions of poor hygiene (Despommier, 2003; Pelloux and Faure, 2004; Smith et al., 2009). Humans are paratenic hosts that acquire infection through accidental ingestion of embryonated eggs (Aguiar-Santos et al., 2004; Mazur-Melewska et al., 2012; Overgaauw and van Knapen, 2013). Larvae hatch in the small intestine and migrate to tissues and organs such as eyes, muscle, liver, lungs, and brain, where they may survive for months to years as larvae (BEAVER et al., 1952; Carvalho and Rocha, 2011; Strube et al., 2013).

Most human infections are asymptomatic, and the development of clinical disease depends on parasite load and the intensity of the host inflammatory response against the larvae. Four clinical forms of toxocariasis are described in humans: visceral larva migrans (LMV), ocular larva migrans (LMO), neurological larva migrans (NLM) and covert (or asymptomatic) toxocariasis (Glickman and Schantz, 1981; Macpherson, 2013). During the acute phase of infection, a complex host immune response is mounted against *Toxocara* spp. larvae involving both innate and adaptive arms of the immune response and resulting in tissue inflammation at the sites of larval invasion. During chronic infections, the larvae induce mechanisms to allow them to survive in the tissues through the release of immune modulatory molecules (Badley et al., 1987; Gasser et al., 2016; Meghji and Maizels, 1986).

Routine laboratory diagnosis of toxocariasis relies on the detection of IgG antibodies to excretory-secretory antigens of *T. canis* (TES) using enzyme-linked immunosorbent assays (ELISA) (Alcantara-Neves et al., 2008; Bojanich et al., 2012; de Savigny and Tizard, 1977). Anthelmintic treatment with albendazole, thiabendazole or ivermectin for toxocariasis is recommended in patients with specific IgG antibodies to *Toxocara* spp. in the presence of relevant clinical manifestations. To date, no vaccine has been developed against toxocariasis. A vaccine would be particularly useful for use in dogs and cats to prevent infection and transmission to humans (Despommier, 2003; Nicholas et al., 1984).

Proteomics has permitted the identification of parasite-derived molecules that can be potentially used to develop new diagnostic tests, vaccines or immunomodulatory agents for the treatment of immune mediated diseases (Huang et al., 2014; Liu et al., 2009; Mutapi, 2012; van der Ree and Mutapi, 2015; Wang et al., 2009b). In the present study, we used a proteomics strategy for profiling the *T. canis* larvae somatic extracts and excreted-secreted products (TES), to identify novel candidates for improved diagnostics, therapeutics and vaccines for human and animal toxocariasis.

**2. Materials and methods**

2.1 Protein sample preparation

*Toxocara canis* eggs were obtained by dissection of uteri from fertile female *Toxocara* adults from infected puppies. Eggs were kept in 3% formalin solution at 25°C for approximately 28 days until embryonation. Larvae were hatched mechanically and maintained at 37ºC in RPMI 1640 medium supplemented with streptomycin (1mg/mL), penicillin (1,000 U/mL), gentamicin (0.2 mg/mL) and amphotericin (2.5µg/mL).

Supernatants were collected at 3-day intervals over three months. Supernatants were supplemented with 0.2 M phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO, USA) and cryopreseved at -70oC. TES proteins were concentrated by ultrafiltration (Millipore Corporate, MA, USA), dialyzed and the protein content estimated using the Bradford method. To obtain the somatic larval extract, third stage larvae were collected from supernatants, washed four times with PBS, and centrifuged at 8000 x g for 10 minutes. The pellet was re-suspended in phosphate saline, pH 7.4 (PBS) and subjected to thermal shock, alternating between liquid nitrogen and heating at 37° C. After this, protease inhibitors (Sigma, St. Louis, Mo.; 2 mM phenylmethylsulfonyl fluoride, 0.2 mM Nα-p-tosyl-l-lysine chloromethyl ketone, 0.2 nM N-tosyl-l-phenylalanine chloromethyl ketone, 25 μg of leupeptin/ml, and 10 mM EDTA) were added and the larvae were ground and the soluble material collected after centrifugation, and stored at -80°C until analysis. The supernatant was stored at -80°C after determination of protein concentration.

2.2 SDS-PAGE of protein extracts and liquid chromatography – tandem mass spectrometry (LC-MS/MS)

TES proteins and larval extracts were separated on 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) at 20 mA (120V) for 2 hours and stained using Comassie Brilliant G250 (Neuhoff et al., 1988). Gel digests were performed with the ProteoExtract® trypsin digestion kit (EMD Millipore) according to the manufacturer’s instructions. Peptides generated by proteolysis were separated by reverse-phase nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Bremen, Germany), loaded onto a trap column (PepSwift Monolithic Trap Column, Dionex) and desalted with 0.1 % (v/v) heptafluorobutyric acid at a flow rate of 10 l/min. After 5 minutes, trap and separation column (PepSwift Monolithic Nano Column, 100 m x 25 cm, Dionex) were coupled with a switching valve and the peptides were eluted with an acetonitrile gradient (Solvent A: 0.1% (v/v) FA/0.01% (v/v) TFA/5% (v/v) ACN; solvent B: 0.1% (v/v) FA/0.01% (v/v) TFA/90% (v/v) ACN; 5–45% B in 60 min) at flow rate of 1 l/min at 55°C. The HPLC was directly coupled via nano electrospray to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Capillary voltage was 2 kV. For peptide identification, a top 12 data-dependent analysis method was used. The instrument was tuned to maximum sensitivity. The normalized fragmentation energy was 27%.

2.3 Data analysis and identification of proteins

Survey and fragment spectra were analyzed with Proteome Discoverer Version 1.4 (Thermo Fisher Scientific) with SequestHT as the search engine and PEAKS Studio 8 (Bioinformatics Solutions, Waterloo, ON, Canada). Searches were done with an in-house database with *T. canis* sequences from UniProtKB ([www.uniprot.org)](http://www.uniprot.org)). Only peptides with high confidence scores (XCorr ≥ 2.3 for SequestHT, −10lgP ≥ 35 for PEAKS) were considered. Blast2GO ([www.blast2go.de/b2ghome](http://www.blast2go.de/b2ghome)) was used for functional classifications of Biological Process, Molecular Function and Cellular Components. Prediction of signal peptides in the identified proteins was done using SignalP v.4.0 (<http://www.cbs.ctu.dk/services/Signal/>).

**3. Results**

3.1 Proteomic profiles of larval excretory/secretory products (TES) and larval extract

The protein profiles of the *T. canis* larval excretory-secretory products and larval extract revealed complex but distinct banding patterns. Proteins were distributed between 12–175 kDa for TES products, whereas those of larval extract were mainly between 10–97 kDa. We identified with high confidence a total of 64 distinct TES proteins and 582 larval extract proteins (Tables 1 and 2; Supplementary 1 and 2). Lists of the main proteins identified with their molecular functions and biological processes are provided in Table 1 and Table 2 for TES and larval extract respectively. The lists contain proteins with 2 or more peptides identified with high confidence and proteins identified in previous studies, containing only one peptide hit.

3.2 Functional annotation of the identified proteins

Functional annotation of the proteins was done using Blast2GO, which revealed structurally and functionally diverse molecules representing proteins whose functions were related to stress responses, reproductive processes, locomotion, response to stimuli, localization, biological regulation, and cellular and metabolic processes (Figures 1). These proteins were present in different families including metalloproteases, metallopeptidases, peptides containing protease inhibition domains, antimicrobial peptides (PAMPS), immunomodulators, nucleases, cytokines, chemokines, digestive enzymes, antibodies, glycine-rich transport proteins, antioxidants such as glutathione S-transferase, phosphatases, and kinases (Tables 1 and 2).

We also identified molecules involved in parasite evasion mechanisms, in addition to those involved in the interaction and modulation of host immune response. Among these proteins, in TES we identified C-type lectin, Tc-ctl-4, CTL-2, phosphatidylethanolamine, collectin 12, cathepsin B-like cysteine proteinase, onchocystatin and serpin (Table 1). In the larval extract, we identified molecules with immunomodulatory properties such as arginase, cystatin, calreticulin, and cathepsins B, L and Z. In both protein preparations, we identified mucin heat shock proteins (HSP), superoxide dismutase and galectin (Tables 1 and 2).

We identified also in the larval extract allergenic proteins such as OV-17 and polyprotein ABA-1 (supplementary Table 1). Furthermore, we found proteins such as troponin, tubulin, actin and ubiquitin, essential for muscular contraction of the parasite (Table 1 and 2). We detected bioproducts in both components of *T. canis* (TES and larval extract), including enzymes involved in metabolic and energy processes such as phosphoglycerate, enolase, glucose-6-phosphate isomerase, phosphoglucomutase, and pyruvate kinase. Among the metalloendopeptidases, superoxide dismutase was present in TES, while aminopeptidase, leukotriene, macrophage migration inhibitory factor-like protein, neprilysin, and neuroserpin were present in the larval extracts.

 **4. Discussion**

4.1 Overview of excretory-secretory products and somatic proteins of *Toxocara canis* larvae proteomics

*Toxocara canis* infection is the most important cause of human toxocariasis, an infection that is of significant public health relevance in human populations with a worldwide distribution (Despommier, 2003; Pelloux and Faure, 2004; Smith et al., 2009). In this study, a proteomic approach allowed us to identify proteins that may potentially be important for parasite survival within the host. *T. canis* infection causes a polarized Th2 immune response profile, associated with enhanced production of cytokines such as IL-4, IL-5 and IL-13, which lead to elevated levels of serum IgE and eosinophilia (Hewitson et al., 2009; Maizels, 2013). A better understanding of the role of *T. canis* molecules in evading the host immune response has implications, both, for the development of vaccines and novel immune modulatory therapies.

To survive in the human host, *Toxocara* spp. larvae employ mechanisms to modulate the host immune response such as by the secretion of molecules that induce the proliferation and activation of regulatory T cells and the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Dlugosz et al., 2015; Długosz et al., 2015; Kuroda et al., 2001; Pinelli et al., 2005). Anti-inflammatory cytokine homologues may actually be released by some helminths to suppress the host inflammatory response (O'Garra et al., 2008; Pinelli et al., 2005). Here we identified several proteins by proteomic analysis of *T. canis* larvae TES and larval extracts that have been shown to have immune modulatory effects such as inhibitors of cysteine protease, onchocystatin, serpin, MIF-like protein and calreticulin. These proteins participate in signal transduction, activation and polarization of cellular immune response pathways. We identified also galectin that has the ability to promote endothelial proliferation, binding to IgE, regulation of the macrophage activation, and inhibition of the T-cell traffic (Bennuru et al., 2009; Hewitson et al., 2008; Kiel et al., 2007; Turner et al., 2008).

4.2 Immunomodulatory molecules

Macrophage migration inhibitory factor (MIF), a cytokine found in humans has homologies with several MIF-like proteins reported in different helminth species that function as modulators of the cellular and humoral immune response. There is evidence that these MIF-like proteins control the inflammatory response through the induction of Treg cells to produce IL-10 and TGF-β (Park et al., 2009; Stavitsky et al., 2003). One of the most representative groups present in the *T. canis* extracts and TES were inhibitors of cysteine proteases, also found in other helminth species such as *Brugia malayi*, *Ascaris lumbricoides* and *Onchocerca volvulus* (Britton and Murray, 2002; Ford et al., 2005; Tort et al., 1999; Winter et al., 2013).

Cysteine proteases have a role in the turnover of the parasite cuticle, and degrade and activate proteolytic enzymes during invasion and migration in the host (Donnelly et al., 2011). Some of these proteins are involved in vital biological processes, such as larval growth and development, tissue degradation, adhesion, migration, molecular communication and differentiation, and parasite evasion mechanisms. Many of the molecules identified are derived primarily from the surface of the parasite or from its excretory/secretory products (Kopitar-Jerala, 2012; Sajid and McKerrow, 2002).

In addition, we detected a cystatin, a reversible cysteine protease inhibitor, homologous to a similar molecule previously identified in *O. volvulus* (Schönemeyer et al., 2001). Cystatin had a molecular weight of 11 kDa and is believed to be involved in the regulation of cysteine protease activity in the parasite, in the modulation of the host immune response, maintaining dendritic cells in an immature state, and compromising the presentation and processing of antigens (Schönemeyer et al., 2001). Recent studies have revealed that cystatins of helminths contribute to downregulation of T-cell proliferation in hosts and induction of anti-inﬂammatory cytokine responses, increasing the production of interleukin 10 (IL)-10 by macrophages (Hartmann and Lucius, 2003; Hewitson et al., 2009; Jang et al., 2011; Zavasnik-Bergant et al., 2005). Previous studies have described the modulatory effect of this molecule from different nematodes such as OV-17 of *O. volvulus* and Av17 of *Acanthocheilonema viteae* (Hartmann et al., 1997; Schönemeyer et al., 2001).

4.3 Cysteine-rich proteins

In this study, we identified important proteases, such as the members of the cysteine protease C1 family, among them cathepsin B, L and Z (present in the larval extract). Cathepsins have been demonstrated in other nematodes with key roles in the modulation of the host immune response, including the proteolytic degradation of the invariant chain of the MHC-II and regulating the intracellular trafficking of this molecule (Williamson et al., 2003). In addition, they participate in antigen processing and cleavage of the intracellular domain of the toll-like receptor (TLR)-9. Suppression of cysteine proteases leads also to the suppression of dendritic cell activation, interference in the formation of the MHC-II peptide antigen complex, and affects the antigen presenting capacity of dendritic cells and the CD4 + T cell response (ten Broeke et al., 2013).

4.4 Heat shock proteins (HSP)

 Another important protein group identified here was the heat shock proteins (HSP) family, present in both *T. canis* larval preparations. HSPs are immunomodulatory molecules, commonly identified in nematodes, which play crucial roles in parasite survival (Narberhaus, 2002; Perez-Morales and Espinoza, 2015; Sotillo et al., 2010). They function as chaperones, facilitating folding and preventing protein aggregation. Furthermore, some authors have suggested that HSPs are immunogenic by stimulating IgG and IgM responses (Dea-Ayuela and Bolas-Fernandez, 2005; Schmitt et al., 2007; Tsan and Gao, 2009). It has been reported that such proteins are potential vaccine targets (Liddell et al., 2003). Several studies have shown that HSPs can induce subsets of regulatory CD4+CD25+ Tregs and stimulate the production of regulatory cytokines, such IL-10 and transforming growth factor-β (TGF-β); therefore, they are candidates as therapeutic agents in allergic and autoimmune diseases (Gaston, 1998; Mansilla et al., 2014; Wang et al., 2009a).

4.5 Structural constituents

In relation to the structural constituents, proteins associated with cytoskeletal and motor activities were identified in *T. canis* larval extract and TES, such as troponin, tubulin, actin and ubiquitin. Actin binds to myosin sites to mediate muscle contraction (Marcilla et al., 2007). Troponin forms a complex that regulates the calcium-dependent interaction of myosin and actin (McArdle et al., 1998; Obinata et al., 2011). These proteins have been reported to be present in other nematodes, (Barstead et al., 1991; Bennuru et al., 2009; Hewitson et al., 2008; Kiel et al., 2007) in addition to *T. canis* (Sperotto et al., 2017).

4.6 Transmembrane and transport

Transmembrane and transport receptor proteins including transthyretin-like protein 46 were identified in both larval components studied. In the larval extract, we identified vitellogenin-6, exportin-1, ferritin, importin-5, phosphoenolpyruvate carboxykinase and transportin-3. In TES, we found apolipophorin, 26 kDa secreted antigen, and collection-12. Transthyretin-like protein, present in *A. suum* and *Caenorhabditis elegans,* is responsible for the transport of retinoic acid and vitamin A (Eneqvist et al., 2003; Vercauteren et al., 2003).

4.7 Evasion mechanisms

The present study also identified *T. canis* surface proteins involved in evasion mechanisms including TES 26 and mucins that coat glycoproteins in the parasite's integument (Gems and Maizels, 1996; Maizels et al., 2000). This latter protein elicits a humoral and cellular immune response with a typical Th2 profile, as well as an innate immune response, with adherence to CDs via the binding of LPS to toll-like receptor (TLR)- 4, and increasing the secretion of IFN-γ and production of IgG antibodies (Dlugosz et al., 2015). It has been shown that this protein may constitute an important target for the development of vaccines against *Fasciola hepatica*, as well as use as diagnostic tool for different nematode infections (Loukas et al., 2000b; Noya et al., 2017).

TES-26, a protein homolog to the family of phosphatidylethanolamine-binding protein (PEBP), is anchored in the plasma membrane and involved in the transport of lipids and cell signalling (Banfield et al., 1998; Gems et al., 1995; Maizels et al., 2000); it is homologous to Sm 14 of *Schistosoma mansoni* (induces immune cross-protection against infection by *S. mansoni*; thus, TES 26 of *T. canis* may be a good vaccine candidate), FABPs of *F. hepatica* and As-p18 of *O. volvulus* (Figueroa-Santiago and Espino, 2014; Thaumaturgo et al., 2002; Zhan et al., 2015). This protein may also play a role in the interaction of parasite ligands with Toll-like receptors of antigen-presenting cells (APCs).

C-Type lectins were also identified in TES and larval extract; they are proteins capable of recognizing carbohydrates associated with glycoconjugates. According to Maizela *et al.* (2013), these molecules are responsible for mediating the inflammatory immune response in tissues and actively participate in the processes of antigen presentation, apoptosis, cell adhesion and polarization of T cells (Hewitson et al., 2009; Loukas et al., 2000a; Loukas et al., 1999; Maizels, 2013). Galectin is another type of lectin linked to galactoside, found in different nematode species, and participates in modulation of the cellular immune response, control of cell adhesion, tumour genesis, apoptosis, inflammatory processes and immune regulation (Turner et al., 2008).

We also found superoxide dismutase and glutathione s-transferase, present on the parasite surface; they participate in evasion mechanisms of the host immune response, neutralizing attack by reactive oxygen species and removing immunomodulatory lipids from the host (Joachim and Ruttkowski, 2008, 2011; Tew and Ronai, 1999; Yim et al., 1993; Zelck and Von Janowsky, 2004). Interestingly, we identified superoxide dismutase, which has anti-inflammatory properties, only in the larval extracts. Superoxide dismutase antagonizes the inflammatory response in the host, protecting the parasite against cell death mediated by the host reactive oxygen species (Bannister et al., 1991; Cardoso et al., 2004; Kim et al., 2000), and is present in various helminths such as *S. mansoni, O. volvulus, Echinococcus granulosus,* *B. malayi,* and *F. hepatica* (Dabir et al., 2008; James et al., 1994; Jang et al., 2011; Kim et al., 2000; Li et al., 2004; Vermeire and Yoshino, 2007).

4.8 Development of the parasite

Several proteins involved in the development of the parasite were detected in the larval extracts, among which was serpin (serine protease inhibitor). In nematode species serpin regulates the proteolytic activity of serine proteases and can be secreted during spermatogenesis of the parasite, besides participating in several immunological processes such as host-parasite interaction, immune evasion by inhibition of neutrophils and cathepsin G (Gettins, 2002; Law et al., 2006). It is homologous to *Anisakis simplex* (Valdivieso et al., 2015), *Schistosoma japonicum* (Molehin et al., 2014) and*B.**malayi* (Zang et al., 1999) serpins.

4.8.1 Energy Metabolism

 We identified many proteins in the larval extracts of *T. canis* involved in energy metabolism related to glycolysis or gluconeogens (glucose-6-phosphate, phosphoenolpyruvate carboxykinase, 2,3-bisphosphoglycerate, phosphoglycerate mutase activity, hexokinase). These molecules actively participate in the energy processes required by the parasite; they can act on the mechanisms of evasion, migration and survival of the parasite within the host. Examples of these were enolases, phosphoenolpyruvate carboxykinase and glyceraldehyde-3-phosphate dehydrogenase; the latter binds to plasminogen, inducing plasmin-mediated proteolysis (Chen et al., 2012; Sotillo et al., 2008). In addition to regulating damage caused by oxidative processes, some authors have described these proteins as immunodominant, probably because they are found on the surface of the parasites, thus interacting directly with the host immune system (Bernal et al., 2006).

4.9 Other functions

 Notably, we found calcium-binding proteins, which were described by Zhu *et. al* (2015) in the transcriptome of *T. canis*, such as annexin, calnexin, calreticulin, calumenin-A in the larval extracts. It has been suggested that helminth annexins are important in the regulation of inflammation, coagulation and intracellular calcium signalling and in the formation and regulation of ion channels, thus representing potential candidates for the development of vaccines against helminth infections (Cross et al., 2016; Gao et al., 2007; Hofmann et al., 2010; Young et al., 2012). Calreticulins in other helminths have been shown to be involved in cellular Ca2+ homeostasis and immune regulation, binding to C1q and inactivating lysis mediated by the classical complement pathway (Coppolino and Dedhar, 1998; Ferreira et al., 2004; Michalak et al., 1999). Mice injected with recombinant calreticulin of *Taenia solium* demonstrated a Th2-modified immune response profile, characterized by the induction of IL-10 in mucosal and systemic lymphoid organs (Rzepecka et al., 2009; Zahreddine et al., 2010).

 Finally, in agreement with the findings of Zhu *et. al.* (2015) we also identified the signaling proteins Ser/Thr protein kinase and protein-tyrosine phosphatase (Zhu et al., 2015). Previous study showed that these molecules are present in several helminths, including in *T. canis* (Ma et al., 2015). They have been implicated in developmental processes such as cell signalling, signal transduction receptors, cell division, ion channel electrophysiology, neurological activity, apoptosis and exocytosis (Forrester et al., 2004; Hoppe et al., 2010; Hu et al., 2007; Klumpp and Krieglstein, 2002).

In conclusion, in this study we used a proteomic approach to identify proteins present in excretory/secretory products and extract of *Toxocara canis* larvae. Among these proteins many are likely to be molecules with important roles in parasite development and survival in the host. We identified proteins likely to be involved in the induction and regulation of the host immune response providing candidate molecules for novel diagnostics, vaccines, and the modulation of the host immune response.

Conflict of interest

There were no conflicting interests that could have influenced the conduct and reporting of this study.

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 **Figure legends**

Fig. 1 - GO pie chart shows representation of the proteins categorized according to Biological process (A and C) and molecular function (B and D) in the TESand larval extract of *T. canis*, respectively.

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| **Table 1.** List of most abundant proteins identiﬁed in TES from *Toxocara canis* by LC-MS/MS   |
| **Description** | **Accession** | **Coverage (%)** | **Peptides** | **MW (kDa)** | **SP** | **Molecular function** | **Biological Process** | **Cellular component** |
| Heat shock 70 kDa  | A0A0B2V5Y2 | 6 | 5 | 101 | No | ATP binding | Unknown function | Unknown function |
| Myosin | A0A0B2W3E0 | 20 | 37 | 23 | No | ATP binding and motor activity | Unknown function | Unknown function |
| Filamin-A  | A0A0B2VIW2 | 1 | 2 | 238 | No | ATP binding and transmembrane receptor protein serine/threonine kinase activity | Unknown function | Integral component of membrane |
| Myosin, essential light chain  | A0A0B2USP9 | 26 | 3 | 21 | No | Calcium ion binding | Unknown function | Unknown function |
| Galectin  | A0A0B2V054 | 8 | 2 | 30 | No | Carbohydrate binding | Galactose metabolic process | Unknown function |
| Excretory/secretory C-type lectin TES-32 | O44927 | 24 | 4 | 23 | Yes | Carbohydrate binding | Unknown function | Unknown function |
| Histone H4 | A0A0B2V2I6 | 63 | 13 | 11 | No | Dna bindin | Nucleosome assembly | Chromosome and nucleus |
| Histone H2B  | A0A0B2UPH8 | 52 | 9 | 13 | No | Dna binding | Unknown function | Nucleosome and nucleus |
| Histone H2A  | A0A0B2UQD6 | 35 | 5 | 13 | No | Dna binding | Unknown function | Nucleosome and nucleus |
| Histone H3 | A0A0B2V2I9 | 60 | 10 | 15 | No | Dna binding | Unknown function | Nucleosome and nucleus |
| Glucose-6-phosphate isomerase  | A0A0B2W5Z0 | 4 | 3 | 67 | No | Glucose-6-phosphate isomerase activity | Gluconeogenesis and glycolytic process | Unknown function |
| Phosphoenolpyruvate carboxykinase [GTP]  | A0A0B2V286 | 4 | 3 | 72 | No | Gtp binding, kinase activity and phosphoenolpyruvate carboxykinase (gtp) activity | Gluconeogenesis | Unknown function |
| Elongation factor 1-alpha  | A0A0B2W5Q7 | 3 | 2 | 50 | No | Gtpase activity, gtp binding and translation elongation factor activity | Unknown function | Cytoplasm |
| Apolipophorin  | A0A0B2VHM0 | 3 | 7 | 349 | No | Lipid transporter activity | Unknown function | Unknown function |
| 26 kDa secreted antigen  | A0A0B2UWT5 | 10 | 3 | 28 | Yes | Lipid binding | Transport | Unknown function |
| Collectin-12  | A0A0B2VX95 | 6 | 2 | 27 | Yes | Lipid binding | Transport | Unknown function |
| **Table 1 (**Continued)  |
| Enolase  | A0A0B2VEA6 | 5 | 2 | 47 | No | Magnesium ion binding and phosphopyruvate hydratase activity | Glycolytic process | Phosphopyruvate hydratase complex |
| Superoxide dismutase [Cu-Zn]  | A0A0B2VI69 | 9 | 3 | 21 | Yes | Metal ion binding and superoxide dismutase activity | Unknown function | Unknown function |
| Phosphoethanolamine N-methyltransferase 1 | A0A0B2VTW9 | 12 | 3 | 30 | No | Methyltransferase activity | Unknown function | Unknown function |
| Paramyosin  | A0A0B2V6Q8 | 2 | 2 | 101 | No | Motor activity | Unknown function | Unknown function |
| Tropomyosin | A0A0B2VDB8 | 29 | 11 | 31 | No | Motor activity | Unknown function | Unknown function |
| Aspartyl protease inhibitor  | A0A0B2V7F9 | 48 | 9 | 16 | No | Peptidase activity | Unknown function | Unknown function |
| Phosphoglycerate kinase  | A0A0B2V4Q8 | 3 | 2 | 66 | No | Phosphoglycerate kinase activity | Glycolytic process | Unknown function |
| Protein disulfide-isomerase  | A0A0B2UJM4 | 12 | 7 | 55 | Yes | Protein disulfide isomerase activity | Cell redox homeostasis | Endoplasmic reticulum |
| Transthyretin-like protein 46 | A0A0B2W0X7 | 34 | 3 | 19 | Yes | Transthyretin-like family protein | Transport | Extracellular space |
| Ancylostoma secreted protein  | A0A0B2UP29 | 8 | 3 | 46 | No | Unknown function | Unknown function | Extracellular region |
| Ancylostoma secreted protein  | A0A0B2VNW7 | 7 | 2 | 27 | No | Unknown function | Unknown function | Extracellular region |
| Major antigen | A0A0B2VV61 | 1 | 3 | 24 | No | Unknown function | Unknown function | Unknown function |
| Macrophage migration inhibitory factor-like protein | A0A0B2V815 | 21 | 3 | 20 | No | Unknown function | Unknown function | Unknown function |
| Proteoglycan core protein  | O76131 | 23 | 4 | 24 | Yes | Unknown function | Unknown function | Unknown function |

The proteins identified were categorized by their molecular function, biological process and cellular component according to information obtained from the Gene Ontology database (UNIPROT).\* Signal peptide.

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| **Table 2.** List of most abundant proteins identiﬁed in larval extract from *Toxocara canis* by LC-MS/MS. (See Supplemental Table 1 for details) |
| **Description** | **Accession** | **Coverage** | **Peptides** | **MW (kDa)** | **SP** | **Molecular function** | **Biological Process** | **Cellular component** |
| 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | A0A0B2VUR0 | 4 | 2 |  64  | No | Phosphoglycerate mutase activity | Glucose catabolic process | Unknown function |
| Actin-2 | A0A0B2URX3 | 19 | 4 |  31  | No | ATP binding | Cytoskeleton organization | Unknown function |
| Alanine aminotransferase 1 | A0A0B2V9Y4 | 6 | 2 |  61  | No | Pyridoxal phosphate binding and transaminase activity | Biosynthetic process | Unknown function |
| Aldehyde dehydrogenase family 9 member A1 | A0A0B2VCZ3 | 5 | 2 |  86  | Yes | Oxidoreductase activity | Unknown function | Unknown function |
| Aldose reductase B | A0A0B2VWR6 | 10 | 2 |  33  | No | Oxidoreductase activity | Unknown function | Unknown function |
| Aminopeptidase N | A0A0B2V4Q4 | 2 | 2 |  213  | Yes | Aminopeptidase activity, metallopeptidase activity and zinc ion binding | Unknown function | Unknown function |
| ANT-3.1 | B5A257 | 24 | 4 |  31  | Yes | Unknown function | Unknown function | Unknown function |
| Apolipoprotein(A) | A0A0B2UXW0 | 20 | 4 |  35  | Yes | Serine-type endopeptidase activity | Unknown function | Unknown function |
| Arginine kinase | A7YVI5 | 9 | 3 |  45  | No | ATP binding and kinase activity | Unknown function | Unknown function |
| Calmodulin  | A0A0B2UVU2 | 20 | 2 |  16  | No | Calcium ion binding | Unknown function | Unknown function |
| Calpain clp-1  | A0A0B2VN55 | 4 | 2 |  61  | No | Calcium-dependent cysteine-type endopeptidase activity | Unknown function | Intracellular |
| Calreticulin | A0A0B2V659 | 11 | 3 |  43  | Yes | Calcium ion binding | Protein folding | Endoplasmic reticulum |
| Calsequestrin | A0A0B2VMM7 | 16 | 3 |  49  | Yes | Calcium ion binding | Unknown function | Unknown function |
| Calumenin-A | A0A0B2VQG1 | 22 | 6 |  36  | Yes | Calcium ion binding | Unknown function | Unknown function |
| Carboxypeptidase | A0A0B2UYY2 | 2 | 4 |  260  | Yes | Serine-type carboxypeptidase activity | Unknown function | Unknown function |
| Carnitine O-acetyltransferase | A0A0B2UYP5 | 7 | 2 |  39  | No | Transferase activity and transferring acyl groups | Unknown function | Unknown function |
| Cathepsin B-like cysteine proteinase 6 | A0A0B2VTL1 | 10 | 2 |  45  | Yes | Cysteine-type peptidase activity | Unknown function | Unknown function |
| Cathepsin L  | A0A0B2VC96 | 6 | 2 |  74  | Yes | Cysteine-type peptidase activity and serine-type endopeptidase activity | Unknown function | Unknown function |
| Cathepsin Z | A0A0B2UNS7 | 4 | 2 |  35  | Yes | Cysteine-type peptidase activity | Unknown function | Unknown function |
| Chaperonin-like protein Hsp-60, mitochondrial | A0A0B2W434 | 5 | 2 |  86  | No | ATP binding | Protein refolding | Cytoplasm |
| Collectin-12  | A0A0B2UY45 | 2 | 2 |  125  | No | Nucleic acid binding | Dna integration | Unknown function |
| C-type lectin protein  | A0A0B2USF5 | 15 | 4 |  43  | Yes | Carbohydrate binding | Unknown function | Unknown function |
| **Table 2 (continued)** |  |  |  |  |  |  |  |  |
| **Description** | **Accession** | **Coverage** | **Peptides** | **MW (kDa)** | **Sp** | **Molecular function** | **Biological process** | **Cellular component** |
| Elongation factor 2 | A0A0B2VI65 | 7 | 3 |  97  | No | Gtpase activity, gtp binding and translation elongation factor activity | Unknown function | Unknown function |
| Enolase  | A0A0B2VEA6 | 29 | 8 | 47 | No | Magnesium ion binding and phosphopyruvate hydratase activity | Glycolytic process | Phosphopyruvate hydratase complex |
| Epidermal retinol dehydrogenase 2  | A0A0B2VCJ7 | 11 | 2 | 38 | No | Oxidoreductase activity | Unknown function | Integral component of membrane |
| Estradiol 17-beta-dehydrogenase 8 | A0A0B2VZ70 | 22 | 4 | 26 | No | Oxidoreductase activity | Unknown function | Unknown function |
| Exportin-1 | A0A0B2UQZ9 | 9 | 7 | 124 | No | Unknown function | Intracellular protein transport | Unknown function |
| Ferritin  | A0A0B2VBR4 | 13 | 2 | 20 | No | Ferric iron binding and ferroxidase activity | Cellular iron ion homeostasis and iron ion transport | Cell |
| Fumarate reductase | A0A0B2W139 | 18 | 6 | 52 | Yes | Oxidoreductase activity | Unknown function | Unknown function |
| Galactokinase | A0A0B2VTC2 | 6 | 2 | 46 | No | ATP binding and galactokinase activity | Galactose metabolic process | Cytoplasm |
| Galectin  | A0A0B2W2G3 | 20 | 2 | 16 | No | Carbohydrate binding | Unknown function | Unknown function |
| Gamma-glutamyltranspeptidase 1 | A0A0B2VJ19 | 5 | 2 | 60 | No | Gamma-glutamyltransferase activity | Glutathione metabolic process | Unknown function |
| Glucose-6-phosphate 1-dehydrogenase | A0A0B2UZ30 | 13 | 5 | 66 | No | Glucose-6-phosphate dehydrogenase activity and nadp binding | Glucose metabolic process and pentose-phosphate shunt | Unknown function |
| Glucose-6-phosphate isomerase | A0A0B2W5Z0 | 25 | 10 | 68 | No | Glucose-6-phosphate isomerase activity | Gluconeogenesis and glycolytic process | Unknown function |
| Glutamate dehydrogenase | A0A0B2UXI4 | 10 | 4 | 59 | No | Glutamate dehydrogenase (nad+) activity | Cellular amino acid metabolic process | Unknown function |
| Glutathione peroxidase  | A0A0B2VSL8 | 6 | 2 | 42 | No | Glutathione peroxidase activity | Response to oxidative stress | Unknown function |
| Glutathione S-transferase  | A0A0B2VSH0 | 10 | 2 | 25 | No | Glutathione transferase activity | Metabolic process | Unknown function |
| Glyceraldehyde-3-phosphate dehydrogenase | A0A0B2UTM8 | 26 | 10 | 44 | No | Glyceraldehyde-3-phosphate dehydrogenase (nad+) (phosphorylating) activity, nad binding and nadp binding | Glucose metabolic process and glycolytic process | Unknown function |
| Heat shock 70 kDa protein  | A0A0B2W0B9 | 9 | 4 | 75 | No | ATP binding | Unknown function | Unknown function |
| **Table 2 (continued)** |
| Heat shock cognate protein HSP 90-beta | A0A0B2VT58 | 28 | 4 | 22 | No | ATP binding | Protein folding and response to stress | Unknown function |
| Heat shock protein HSP 90-alpha | A0A0B2V484 | 26 | 13 | 75 | No | ATP binding | Protein folding and response to stress | Unknown function |
| Hemicentin-1 | A0A0B2UNR1 | 6 | 3 | 131 | No | Unknown function | Unknown function | Unknown function |
| Hexokinase | A0A0B2USB4 | 16 | 5 | 52 | No | ATP binding, glucose binding and hexokinase activity | Cellular glucose homeostasis and glycolytic process | Cell |
| Immunoglobulin-binding protein 1 | A0A0B2VJL5 | 18 | 4 | 39 | No | Regulation of signal transduction | Unknown function | Unknown function |
| Importin-5 | A0A0B2VLS9 | 12 | 10 | 126 | No | Unknown function | Intracellular protein transport | Intracellular |
| Inositol monophosphatase | A0A0B2USQ4 | 7 | 2 | 37 | No | Inositol monophosphate 1-phosphatase activity | Inositol phosphate dephosphorylation source | Unknown function |
| Isocitrate dehydrogenase [NAD] subunit gamma | A0A0B2VSC5 | 2 | 2 | 121 | Yes | Isocitrate dehydrogenase (nad+) activity, magnesium ion binding, nad binding and serine-type endopeptidase activity | Lipid metabolic process and tricarboxylic acid cycle | Unknown function |
| Leukotriene A-4 hydrolase | A0A0B2VFU8 | 10 | 4 | 71 | No | Metallopeptidase activity and zinc ion binding | Unknown function | Unknown function |
| Macrophage migration inhibitory factor-like protein | A0A0B2V815 | 34 | 4 | 21 | No | Unknown function | Unknown function | Unknown function |
| Major allergen Ani s 1  | A0A0B2V5M2 | 13 | 2 | 21 | Yes | Serine-type endopeptidase inhibitor activity | Unknown function | Unknown function |
| Major pepsin inhibitor | A0A0B2VB99 | 16 | 2 | 17 | No | Unknown function | Unknown function | Unknown function |
| Major sperm protein  | A0A0B2VCS9 | 9 | 2 | 25 | No | Unknown function | Unknown function | Cytoskeleton |
| Mesocentin | A0A0B2VWQ7 | 3 | 4 | 233 | No | Unknown function | Unknown function | Unknown function |
| Microsomal triglyceride transfer protein large subunitSV=1 | A0A0B2VPL4 | 3 | 2 | 113 | Yes | Lipid transporter activity | Unknown function | Unknown function |
| Myoglobin | A0A0B2VPY5 | 12 | 2 | 17 | No | Heme binding, iron ion binding, oxygen binding and oxygen transporter activity | Unknown function | Unknown function |
| Neprilysin-1 | A0A0B2VJQ9 | 7 | 19 | 424 | No | Metalloendopeptidase activity | Unknown function | Unknown function |
| Neuroserpin | A0A0B2V666 | 3 | 2 | 120 | No | Unknown function | Unknown function | Extracellular space |
| Onchocystatin  | A0A0B2V581 | 36 | 4 | 22 | Yes | Cysteine-type endopeptidase inhibitor activity | Unknown function | Unknown function |
| **Table 2 (continued)** |  |  |  |  |  |  |  |  |
| OV-16 antigen O | A0A0B2V438 | 55 | 6 | 19 | No | Unknown function | Unknown function | Unknown function |
| OV-17 antigen  | A0A0B2VKQ1 | 54 | 10 | 15 | Yes | Unknown function | Unknown function | Unknown function |
| Paramyosin | A0A0B2UZA1 | 16 | 3 | 33 | No | Motor activity  | Unknown function | Myosin complex |
| Phosphatidylethanolamine-binding-like protein F40A3.3  | A0A0B2VUI6 | 39 | 4 | 20 | No | Unknown function | Unknown function | Unknown function |
| Phosphatidylinositol | A0A0B2VF93 | 8 | 2 | 50 | No | Phosphatidylinositol phosphate kinase activity | Unknown function | Unknown function |
| Phosphatidylinositol phosphatase  | A0A0B2VJK9 | 1 | 2 | 449 | Yes | Unknown function | Unknown function | Integral component of membrane |
| Phosphoenolpyruvate carboxykinase | A0A0B2UWV2 | 12 | 5 | 76 | No | Transport | Unknown function | Intracellular |
| Phosphoenolpyruvate carboxykinase [GTP] | A0A0B2V286 | 29 | 13 | 72 | No | Gtp binding, kinase activity and phosphoenolpyruvate carboxykinase (gtp) activity | Gluconeogenesis | Unknown function |
| Plasminogen | A0A0B2V8X7 | 14 | 3 | 32 | Yes | Serine-type endopeptidase activity | Unknown function | Unknown function |
| Pyruvate kinase | A0A0B2URT1 | 13 | 7 | 74 | No | Kinase activity, magnesium ion binding, potassium ion binding and pyruvate kinase activity | Unknown function | Unknown function |
| Quinone oxidoreductase-like protein 2-like protein | A0A0B2VLU5 | 23 | 4 | 37 | No | Oxidoreductase activity and zinc ion binding | Unknown function | Unknown function |
| Reticulocyte-binding protein 2-like protein a | A0A0B2VVR5 | 6 | 2 | 74 | No | Unknown function | Unknown function | Unknown function |
| Reticulon-like protein  | A0A0B2VR17 | 12 | 2 | 29 | No | Unknown function | Unknown function | Endoplasmic reticulum membrane  |
| Serine hydroxymethyltransferase | A0A0B2W560 | 11 | 4 | 55 | No | Glycine hydroxymethyltransferase activity, methyltransferase activity and pyridoxal phosphate binding | Glycine metabolic process source: interprol-serine metabolic process source: interprotetrahydrofolate interconversion | Unknown function |
| Serpin B6 | A0A0B2VH56 | 8 | 2 | 44 | Yes | Unknown function | Unknown function | Extracellular space |
| Sorbitol dehydrogenase  | A0A0B2VJ07 | 10 | 2 | 39 | No | Oxidoreductase activity and zinc ion binding | Unknown function | Unknown function |
| Spermidine synthase | A0A0B2V470 | 11 | 4 | 40 | No | Transferase activity | Polyamine metabolic process | Unknown function |
| Superoxide dismutase [Cu-Zn]  | A0A0B2UYA2 | 18 | 2 | 19 | Yes | Metal ion binding and superoxide dismutase activity | Unknown function | Unknown function |
|  |  |  |  |  |  |  |  |  |
| Thyrotropin-releasing hormone-degrading ectoenzyme | A0A0B2UZP1 | 5 | 7 | 215 | No | Metallopeptidase activity and zinc ion binding | Unknown function | Unknown function |
| Thyrotropin-releasing hormone-degrading ectoenzymeSV=1 | A0A0B2V4Y5 | 2 | 3 | 216 | Yes | Metallopeptidase activity and zinc ion binding | Unknown function | Unknown function |
| Titin | A0A0B2VJV3 | 3 | 3 | 166 | No | Unknown function | Unknown function | Unknown function |
| Transportin-3 | A0A0B2VBU8 | 5 | 3 | 103 | No | Unknown function | Unknown function | Unknown function |
| Transthyretin-like protein 46  | A0A0B2VAW6 | 19 | 2 | 17 | Yes | Unknown function | Unknown function | Extracellular space |
| Tropomyosin | A0A0B2VDB8 | 27 | 7 | 31 | No | Unknown function | Unknown function | Unknown function |
| Troponin I  | A0A0B2VTU0 | 9 | 2 | 31 | No | Unknown function | Unknown function | Troponin complex |
| Troponin T | A0A0B2USX2 | 7 | 3 | 44 | No | Unknown function | Regulation of muscle contraction | Troponin complex |
| Tubulin alpha chain | A0A0B2VPL2 | 25 | 7 | 50 | No | Gtpase activity, gtp binding and structural constituent of cytoskeleton | Microtubule-based process | Cytoplasm and microtubule |
| Twitchin | A0A0B2UWL8 | 19 | 15 | 140 | No | ATP binding and protein kinase activity | Unknown function | Unknown function |
| Ubiquitin-conjugating enzyme E2 variant 2 | A0A0B2VYL2 | 45 | 6 | 16 | No | Unknown function | Unknown function | Unknown function |
| Vitellogenin-6 | A0A0B2V8F3 | 54 | 86 |  198  | Yes | Lipid transporter activity | Unknown function | Unknown function |

The proteins identified were categorized by their molecular function, biological process and cellular component according to information obtained from the Gene Ontology database (UNIPROT).\* Signal peptide.