Published in final edited form as:

J Proteomics. 2018 March 20; 175: 127–135. doi:10.1016/j.jprot.2017.12.022.

Comparative proteomics of the larval and adult stages of the model cestode parasite *Mesocestoides corti*☆

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Abstract

Mesocestoides corti is a widely used model for the study of cestode biology, and its transition from the larval tetrathyridium (TT) stage to the strobilated, adult worm (ST) stage can be induced and followed *in vitro*. Here, a proteomic approach was used to describe and compare *M. corti* TT and ST protein repertories. Overall, 571 proteins were identified, 238 proteins in TT samples and 333 proteins in ST samples. Among the identified proteins, 207 proteins were shared by TTs and STs, while 157 were stage-specific, being 31 exclusive from TTs, and 126 from STs. Functional annotation revealed fundamental metabolic differences between the TT and the ST stages. TTs perform functions related mainly to basic metabolism, responsible for growth and vegetative development by asexual reproduction. STs, in contrast, perform a wider range of functions, including macro-molecule biosynthetic processes, gene expression and control pathways, which may be associated to its proglottization/segmentation, sexual differentiation and more complex physiology. Furthermore, the generated results provided an extensive list of cestode proteins of interest for functional studies in *M. corti*. Many of these proteins are novel candidate diagnostic

☆The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC). Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the U.S. Department of Health and Human Services.

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Conflict of interest

Authors declare no conflicts of interest.

antigens, and/or potential targets for the development of new and more effective antihelminthic drugs.

Biological significance: Cestodiases are parasitic diseases with serious impact on human and animal health. Efforts to develop more effective strategies for diagnosis, treatment or control of cestodiases are impaired by the still limited knowledge on many aspects of cestode biology, including the complex developmental processes that occur in the life cycles of these parasites. *Mesocestoides corti* is a good experimental model to study the transition from the larval to the adult stage, called strobilation, which occur in typical cestode life-cycles. The performed proteomics approach provided large-scale identification and quantification of *M. corti* proteins. Many stage-specific or differentially expressed proteins were detected in the larval tetrathyridium (TT) stage and in the strobilated, adult worm (ST) stage. Functional comparative analyses of the described protein repertoires shed light on function and processes associated to specific features of both stages, such as less differentiation and asexual reproduction in TTs, and proglottization/segmentation and sexual differentiation in ST. Moreover, many of the identified stage-specific proteins are useful as cestode developmental markers, and are potential targets for development of novel diagnostic methods and therapeutic drugs for cestodiases.

Keywords

Cestode; Development; Strobilation; Segmentation; Parasite; MS/MS proteomics

1. Introduction

Cestodes (tapeworms) are obligatory parasites and exhibit diverse adaptations to their parasitic lifestyle [1]. These adaptations include some evolutionary innovations in development, such as complex life-cycles, including two or more hosts, different life-stages specialized to invade and survive in different host species, mechanisms of asexual reproduction, and body segmentation, with the serial repetition of reproductive structures as a mean to generate massive progenies [1,2]. Therefore, cestode biology is unique in many aspects and the studies of their developmental processes are important not only for the understanding of adaptations to parasitism, but also to provide molecular bases for the development of novel strategies for prevention, control and/or treatment of cestodiases.

Cestode species exhibit a diversity of life cycles and developmental strategies [3]. However, some characteristics are common in most species, and a general life-cycle can be schematized [2,4]. In a typical cestode life cycle, the adult worm resides in the intestine of a vertebrate host (the definitive host). Adult cestodes are usually hermaphrodites and generate egg by sexual reproduction. Each egg undergoes embryonic development to generate an oncosphere, which is released in the environment. The egg infects a suitable intermediate host species, where it hatches, and the oncosphere undergoes metamorphosis, developing into the next life-stage, the metacestode larva. The metacestode is eventually ingested by the definitive host, and once in the host's intestine, it develops into the adult stage, segmented and sexually mature, thus completing the life-cycle.

The metacestode development into the adult worm, includes the larva body (strobilum) formation, and is called strobilation [2]. It is considered by some authors as resulting from

two independent events: (i) proglottization, the serial repetition of the gonads; and (ii) segmentation, the external division of the proglottids [5]. Therefore, a typical strobilation process results in the production of tandem reproductive units exhibiting progressive degrees of maturation. This is a notable feature of the biology of cestode, found in all Eucestoda subclass, to which belong *M. corti* and several species of relevance as parasites of human and domestic animals, like those from genuses *Echinococcus*, and *Taenia*.

Tapeworms are able to infect an extraordinary broad diversity of animal hosts. Cestode species infect virtually any vertebrate species, and they are easily transmitted between hosts [1]. Diseases caused by cestodes (cestodiases) affect both human and domestic animals worldwide [6–8], with profound social and economic consequences [9,10]. Echinococcosis and cysticercosis, for instance, are major cestodiases, whose etiological agents are the larval forms (metacestodes) of *Echinococcus* spp. and *Taenia* spp., respectively. Due to their impact in public human health, these cestodiases were included by the World Health Organization in its list of neglected tropical diseases (http://www.who.int/neglected_diseases/en/). Although many efforts have been made to control these cestodiases, they persist as endemic or reemergent diseases, nearly worldwide [11]. The search for more refined methods for diagnosis, treatment and control of cestodiases is limited by the still poor knowledge of the biology of these parasites, especially about the molecular biology of their developmental processes [12,13].

Mesocestoides corti (syn *M. vogae*) is a broadly used model for cestode biology [14–16]. Its life cycle requires one definitive host and two intermediate hosts [17]. The *M. corti* metacestode larva, the tetrathyridium (TT), lives as a cyst in the liver or free in the peritoneal cavity of the second intermediate host (which may be a mammal, a bird, an amphibian, or a reptile). There, tetrathyridia (TTs) massively replicate by asexual reproduction and slowly colonize the host. Upon ingestion by the definitive host (canids, felids, or a mustelid), a TT undergoes a typical strobilation process to generate a strobilated adult worm (ST), sexually differentiated and with typical cestode proglottization/segmentation [18].

M. corti TTs can be easily multiplied *in vivo*, in experimental hosts, where they reproduce asexually, and their process of maturation into sexually differentiated and segmented STs can be induced and followed *in vitro* [19,20]. Moreover, *M. corti* STs produced *in vitro* can be safely manipulated, as the eventually produced eggs/oncospheres are not infective to humans [20].

Different approaches have been used to study *M. corti* strobilar development *in vitro*. Conditions to efficiently induce tetrathyridium strobilar development and reach high rates of fully strobilated worms have been standardized by our group [20,21]. The standardized *in vitro* conditions have then been used for both *M. corti* transcriptomics and proteomics studies in order to identify differentially expressed genes and proteins between TTs and STs. Pioneer transcriptomic studies using subtracted cDNA libraries and microarrays [22], and, more recently, RNA-seq approaches, have been carried out to describe mRNA and miRNA repertoires of both *M. corti* TTs and STs [23] (Basika et al., manuscript in preparation). Complementary proteomics studies, also carried out by our group, allowed to generate the

first descriptions of the protein repertoires from *bona fide* TTs [24,25], from TT excretion/secretion products [25], and from TTs 24 h after strobilation induction [24].

Early *M. corti* transcriptomics and proteomics studies have relied on EST and/or genome sequences from related species, such as *Echinococcus* spp. and *Taenia* spp., to identify *M. corti* transcripts or proteins. More recently, however, a draft version of the *M. corti* genome sequence became available from the Sanger Institute '50 Helminth Genomes Project' (http://www.sanger.ac.uk/science/collaboration/50hgp) [26], improving RNA and protein identification on the more recent *M. corti* 'omics' studies, including the comparative proteomics study described here.

This study describes a large-scale comparative proteomics analysis of *M. corti* TTs and STs. Comprehensive descriptions of the protein content of both stages are provided, significantly incrementing the knowledge on the composition and abundance of proteins present in TTs and/or in STs. The generated results provide useful functional information on cestode molecular, and cellular processes, associated to survival and development of the parasites. Moreover, potential developmental markers, and candidate target molecules for the future development of new and more efficient methods for diagnosis or treatment of tapeworm infections are discussed.

2. Materials & methods

2.1. Collection and cultivation of Mesocestoides corti

M. corti metacestode larvae (TT) were maintained *in vivo* by serial passages in experimental hosts (BALB/c mice and Wistar rats), as described by Markoski et al. [20]. The procedures involving animals were previously approved by the Ethical Committee (CEUA) of the Universidade Federal do Rio Grande do Sul (Project no. 25726).

In vitro culture of TTs and production of STs were carried out essentially as previously described [20,21]. Briefly, TTs obtained from mice were cultured in RPMI 1640 medium at 37 °C in an atmosphere of 5% CO_2, for 24 h. After this period, TT samples were washed and stored in phosphate buffered saline (PBS) at −80 °C. To obtain ST samples, 24-h cultured TTs were treated with trypsin (10^5 units BAEE/ml in RPMI medium supplemented with 10% fetal bovine serum) for 24 h in an atmosphere of 5% CO_2 at 39 °C to induce strobilation. Induced TTs were then cultured in RPMI medium supplemented with 20% fetal bovine serum in an atmosphere of 5% CO_2 and 39 °C, and the progression of strobilation was monitored daily by inspection in an optical microscope. A worm was considered fully strobilated upon microscopic confirmation of segmentation and proglottization, with the differentiation of internal sexual organs in each proglottid. For any given culture, strobilation efficiency was measured as the ratio of fully strobilated worms to the total number of individuals. Cultures for the production of TT and ST samples for proteomics were all performed in triplicates (biological replicates), and only cultures with strobilation efficiency > 70% were used to produce ST samples. ST samples were washed and stored in PBS at −80 °C.

2.2. Sample preparation for mass spectrometry analysis

TT and ST samples were washed seven times with 100 mM Tris-Cl buffer (pH 8.5) for removal of culture debris. Parasite samples were suspended in 200 μL of 0.1% RapiGest SF Surfactant (Waters) and cell lysis was performed by sonication (Sonicator Q500, Qsonica) in ice, using 5 pulses (30% amplitude) of 30 s each and 2 min interval between pulses. The lysates were clarified by centrifugation (15,000 × *g* for 30 min) at 4 °C. Sample quality was assessed by 12% SDS-PAGE; a volume of 8 μL of each sample was loaded onto the gel. Thermo Scientific Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific) was used to quantify proteins in each sample. After quantification, 100 μg of protein from each sample were used for the liquid chromatography/mass spectrometry (LC-MS/MS) analysis.

Samples were in-solution digested according to the RapiGest SF Surfactant protocol, using trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) at a ratio of 1 μg of enzyme:100 μg of protein. After digestion for 4 h at 37 °C, an additional aliquot (1 μg of enzyme:100 μg of protein) of the enzyme was added, and samples were incubated for additional 16–20 h at 37 °C. RapiGest SF Surfactant removal after digestion was carried out with 0.5% trifluoroacetic acid (TFA) (v:v). The resulting peptides were desalted using an OASIS® HLB Cartridge (Waters). Briefly, columns were conditioned with 2 mL of 100% acetonitrile and equilibrate with 2 mL of 0.1% trifluoroacetic acid (TFA). The samples were added to the columns and an additional aliquot of 2 mL of 0.1% TFA was added to the columns to wash out any trace of salts. The peptides were eluted with 300 μL of 50% acetonitrile (ACN)/0.1% TFA. The desalted peptides were vacuum dried and reconstituted in 5 mM KH_2PO_4 (pH 3.0)/25% ACN for SCX fractionation.

The peptides were fractionated using a PolySULFOETHYL Aspartamide™ SCX Minispin Column (Harvard Apparatus). SCX spin columns were conditioned with 100 μL of 100% methanol, washed twice with 100 μL of milli-Q water and left to stand in 100 μL of 0.2 M monosodium phosphate/0.3 M sodium acetate for at least 1 h. The columns were then equilibrated with 100 μL of 5 mM KH_2PO_4 (pH 3.0)/25% ACN buffer prior to sample loading. After two washes with equilibration buffer, peptides were then eluted in a stepwise gradient of increasing salt concentration (100, 175, 250, 325 and 400 mM KCl) in 50 μL of equilibration buffer. Desalting of each SCX fraction was performed with OASIS® HLB Cartridges (Waters) as mentioned before. Samples were dried in a vacuum concentrator and stored at −20 °C until LC-MS/MS analysis.

2.3. Mass spectrometry analysis

LC-MS/MS analyses were performed essentially as previously reported by Debarba et al. [27]. Briefly, each SCX fraction was reconstituted using 0.1% formic acid (FA) (Thermo Scientific, Rockford, IL) in water and loaded onto a nanoAcquity UPLC system (Waters Corporation, Milford, MA). Solvents A and B, 0.1% FA in water and 0.1% FA in acetonitrile (Burdick and Jackson), respectively, were used in the mobile phase. The gradient flow was set at 300 nl/min. The gradient consisted of a hold at 5% B for 5 min, followed by a ramp up to 35% B over 25 min, then a ramp up to 95% B in 5 min, a hold at 95% B for 5 min before returning to 5% B in 5 min and re-equilibration at 5% B for 20 min. Peptides were analyzed in an Orbitrap Elite tandem mass spectrometer (Thermo Scientific, San Jose, CA). A 2.0

kV voltage was applied to the nano-LC column. The mass spectrometer was programmed to perform data-dependent acquisition by scanning the mass range from mass-to-charge (*m*/*z*) 400 to 1600 at a nominal resolution setting of 60,000 for parent ion acquisition in the Orbitrap. For the MS/MS analysis, the 15 most intense ions with two or more charges were isolated and fragmented in a second round of MS.

2.4. Database search and protein identification parameters

MS/MS raw data were processed using the msConvert tool (ProteoWizard, version 3) [28], and the peak lists were exported in the Mascot Generic Format (.mgf). Database search was performed using Mascot Search Engine (Matrix Science, version 2.3.02). All protein searches were performed using the deduced amino acid sequences from the *M. corti* genome assembly version 1.0.4 annotation available in the WormBase ParaSite (http://parasite.wormbase.org, Helminth Genomes Consortium). Fragment ion mass tolerance of 1 Da and peptide ion tolerance of 10 ppm were used. Carbamidomethylation of cysteines was specified as a fixed modification, whereas the oxidation of methionine was specified as a variable modification.

To validate protein identifications, MS/MS data were analyzed in Scaffold (Proteome Software Inc., version 4.4.1.1). MASCOT *.dat files of SCX fractions derived from the same biological sample were loaded on Scaffold using Multidimensional Protein Identification Technology (MudPIT) option [29]. Peptide identifications were accepted if they could be established at > 95% of probability as assigned by the Peptide Prophet algorithm [30] and the protein identifications were accepted if they could be established at > 99% of probability as assigned by the Protein Prophet algorithm [31] and contained at least 2 identified peptides. The false discovery rate, FDR (Decoy), was < 1% for proteins and peptides. The normalized spectral abundance factor (NSAF) [32] was calculated for each protein using Scaffold. Quantitative differences between TT and ST NSAF values were statistically analyzed using Student's *t*-test and p-value correction with the Benjamini & Hochberg false discovery rate (FRD); values of $p < .05$ were considered statistically significant. The heat map was performed with NSAF values normalized by Z-score calculation; hierarchical clustering was used as method of cluster analysis and the distance between proteins was calculated by Euclidean distance. The t-test and heat map analyses were performed in Perseus software (MaxQuant, version 1.5.5.1.) [33]. Volcano plot analysis, to demonstrate the magnitude, fold change (FC), and significance of quantitative differences, were performed in Scaffold. Proteins with FC > 1, and $p < .05$ (p-value correction with the Benjamini & Hochberg - FRD), were considered differentially represented between compared samples and numbered in the volcano plot.

2.5. Functional annotation and gene ontology (GO) term enrichment analysis

Functional annotation and GO term enrichment analysis for differentially abundant proteins were performed using the Cytoscape plugin BiNGO 3.0.3 [34] through a customized search. The files associated with *M. corti* protein annotation data were courteously provided by Wellcome Trust Sanger Institute (UK), while the ontology files were retrieved from the GO database [35]. Functional enrichment analysis were performed using hypergeometric distribution and p-value correction with the Benjamini & Hochberg false discovery rate

(FRD). Values of $p \leq .05$ were considered statistically significant [34]. The resulting lists of enriched GO terms were summarized by removing redundant GO terms using REVIGO [36]. The semantic similarity of the GO terms was calculate through SimRel (default allowed similarity = 0.7) and the results were plotted using R [37]. The KAAS server [38] was used to map KEGG pathways; the bi-directional best hit method was used to assign orthologues (threshold of BLAST bit scores = 50). The representative gene data set for eukaryotes plus those for *Schistosoma mansoni* were used as references in KAAS mapping.

3. Results

3.1. LC-MS/MS identification of proteins in M. corti TT and ST samples

Protein content and quality of three different samples (biological replicates) of *M. corti* TTs and STs were initially assessed by 12% SDS-PAGE analysis. TT and ST samples showed a complex mixture of proteins ranging from 225 kDa to < 10 kDa (Fig. S1). Nearly identical electrophoretic profiles were evident for the biological replicates from both TT and ST samples, which was taken as evidence of the expected experimental/sample reproducibility. TT and ST samples also presented roughly similar electrophoretic profiles, but with some differences in the number of protein bands. Additional differences were also observed when the relative intensities of the protein bands are compared within and between samples.

TT and ST protein extracts were analyzed by LC-MS/MS and the repertoires of identified TT and ST proteins were compared to each other for identification of shared and stage-exclusive proteins. To assure confidence and reproducibility, only proteins with at least two peptides and present in at least two of the three biological replicates of each sample (TT or ST) were considered for protein identification. A summary of the LC-MS/MS results is presented in Fig. 1, and the full lists of identified proteins are shown in Table S1A–C; the list of detected peptides is in Table S2. Overall, 571 *M. corti* proteins were detected, 238 in TT samples, and 333 proteins in ST samples, respectively. A total of 207 proteins (56.9%) were shared between TT and ST samples, while 31 (8.5%) and 126 (34.6%) were exclusively detected in the TT and ST stages, respectively.

From the 207 proteins shared between the TT and ST stages, a set of 159 did not present any statistically significant difference in abundance ($p \geq .05$) between parasite stages based on NSAF quantitative data (Table S1A). In this set there are energetic metabolism-related enzymes (*e.g.* phosphoenolpyruvate carboxykinase, glyceraldehyde-3-phosphate dehydrogenase, and glycogen debranching enzyme), structural proteins (*e.g.* adducin-related protein 1, actin-related protein, and moesin/ezrin/radixin homolog 1), extracellular matrix structural constituents (*e.g.* collagen alpha-1(V) chain, cuticle collagen 75, and cuticle collagen 14), enzymes related to protein metabolism, and modification (*e.g.* endoplasmic reticulum metallopeptidase, calpain-C, and peptidase C1A), chaperones (*e.g.* heat shock 70 protein A, heat shock 70 protein F, and heat shock 90), cell regulatory proteins (including 14-3-3 proteins, dynamin, ras-related C3, atrophin-1, glucose-regulated protein, and disulfide-isomerase 1), and two isoforms of a putative fatty acid binding protein (FABP).

3.2. Differentially represented proteins in TT and ST samples

The repertoire of TT exclusive proteins (31 proteins; 8.5% of the total of identified proteins) (Table S1B), include metabolic proteins (plasma alpha-L-fucosidase, L-lactate dehydrogenase, and glycoside hydrolase), signal transduction proteins (ras protein let-60, ras-related protein rab-17, ras-related protein ab-15, integrin alpha-5 and uncharacterized integrin beta-like protein) and regulatory proteins (PP1 and PP2A). At least some of these stage-specific proteins may represent TT-specific developmental markers.

The number of ST exclusive proteins (126 proteins) was 4 times larger than that found for TTs, corresponding to 34.6% of total identified proteins (Table S1C). This repertory includes several proteins related to gene expression, protein expression and turnover, such as proteins with transcription cofactor activity (SNase-like, and TSN-1); translation initiation factors (eukaryotic translation initiation factor 2 subunit 3, and eukaryotic translation initiation factor 3 subunit K); ribosomal proteins (40S ribosomal protein S11, 60S acidic ribosomal protein P1, 60S ribosomal protein L8, and others); tRNA synthetases (putative aspartyl-tRNA synthetase, and putative glutamyl-tRNA synthetase), and tRNA ligases (cysteine-tRNA ligase, lysine-tRNA ligase, probable arginine-tRNA ligase, and threonine-tRNA ligase); chaperone proteins (peptidyl-prolyl cis-trans isomerase, endoplasmin and T-complex proteins); and proteolysis (mitochondrial lon protease homolog, and CAAX prenyl protease 1 homolog).

Quantitative data (NSAF values) were also used to define the repertoires of shared proteins with significantly different abundance between TTs and STs. Forty eight (23.18%) out of 207 shared proteins were considered differentially represented between the compared parasite's life-stages ($p \leq .05$, Table S1), and were represented in a heat map (Fig. S2). From these proteins, 27 were found overrepresented in TTs, and 21 were found overrepresented in STs (Table S1A). A volcano plot analysis was performed in order to investigate the magnitude of the differences in protein abundance between TTs and STs (Fig. 2). In this analysis, proteins with a p value $< .05$ and a FC > 1 were considered differentially abundant between TTs and STs by both statistical and FC parameters. Twenty-eight proteins satisfied these criteria, which were identified in Fig. 2.

Among the 27 shared proteins with higher abundance in TTs (Table S1A), there are proteins with intracellular signaling activity (two 14-3-3 protein isoforms), binding activity (LIM-9 - zinc ion binding, low-density lipoprotein receptor - lipid binding, and spectrin beta chain - phospholipid binding), oxidoreductase activity (steroid dehydrogenase, and L-lactate dehydrogenase), phosphorylase activity (glycogen phosphorylase), structural proteins (tubulin alpha-4A chain, and tubulin alpha-2 chain), and one hypothetical protein. According to the volcano plot analysis, proteins associated with embryonic morphogenesis (papilin, FC 2.41), locomotion (paramyosin, FC 1.42), and with kinase regulator activity (Prkar2, FC 1.78) were also found overrepresented ($p < .05$, FC > 1) in TTs.

Among the 21 shared proteins with higher abundance in STs (Table S1A), there are proteins with nucleic acid binding activity (histone H4-like protein type G, 40S ribosomal protein S2, putative 60s ribosomal protein L4, and 40S ribosomal protein S7), redox activity (SDH1, and dihydrolipoyl dehydrogenase), transmembrane transport (putative ADP, ATP carrier

protein, and sideroflexin), and metabolic activity (2-oxoglutarate dehydrogenase, and malate dehydrogenase). According to the volcano plot analysis, proteins associated with basic metabolism (pyruvate dehydrogenase, FC 1.78; and GCAT KBL, FC 2.55); and nucleic acid synthesis (pol-related, FC 1.03), and protein folding (heat shock protein 60 kDa, FC 1.11; and T-complex protein 1 subunit (TCP1β; FC 2.50) were also found overrepresented ($p < .05$, $FC > 1$) in STs.

3.3. Functional annotation of differentially abundant proteins between TT and ST samples

KEGG pathway analyses using KAAS server were performed to annotate biochemical pathways for differentially abundant proteins (including stage-specific proteins shared proteins with significant difference in abundance between TT and ST samples). The KEGG codes assigned to the differentially abundant proteins detected in TTs and STs are in Table S3. From the total of 205 proteins addressed in the analysis, 145 could be assigned to at least one biochemical pathway, 36 in TTs (Table S3A), and 109 in STs (Table S3B). These 145 proteins were assigned to a total of 151 unique pathways, 94 in TTs, and 118 in STs (Table S3C and D, respectively). 61 (40.4%) of these pathways are common to both TTs and STs, while 33 (21.9%) of them were found only for TTs, and 57 (37.7%) were found only for STs. The top 10 TT and ST assignments of differential proteins shown in Table 1 (which include pathways with a minimum of 4 assignments) revealed marked differences between these two stages. They differ in 6 of them, sharing only the top four pathways, related to basic metabolism. Differential top 10 biochemical pathways in TTs include “regulation of actin cytoskeleton”, and “Starch and sucrose metabolism” pathways and also some signaling pathways, like “insulin signaling pathway”, “PI3K-Akt signaling pathway” and “Meiosis”. On the other hand, differential top 10 biochemical pathways in STs, include “Ribosome”, “Carbon metabolism”, “Citrate cycle (TCA cycle)”, “Oxidative phosphorylation”, and “Aminoacyl-tRNA biosynthesis”.

Additional functional enrichment analyses were performed for the whole set of differentially abundant proteins between TT and ST samples (205 proteins). Functional classification and gene ontology (GO) enrichment data for the TT and ST differentially abundant proteins are shown in Tables S4 and S5. Most proteins were functionally annotated for both TT (51 out of 58 proteins; 87.93%) and ST (133 out of 147 proteins; 90,47%); no functional annotation was attributed to only 7 TT proteins, and to 14 ST proteins (Table S4A and B, respectively). Functional enrichment ($p \leq .05$) was found for 28 GO subcategories in TTs, and for 95 GO subcategories in STs. The GO terms for TT and ST differential proteins were summarized by REVIGO and the complete lists of summarized non redundant terms are shown in Table S5, and graphically represented in Fig. 3.

As can be shown in Fig. 3A, five REVIGO category clusters are enriched in TT samples, namely (from the more to the less enriched) “carbohydrate metabolic process”, “cellular carbohydrate metabolic process”, “mitotic spindle organization”, “ADP metabolic process”, and “generation of precursor metabolites and energy”. On the other hand, as shown in Fig. 3B, 15 REVIGO category clusters were found enriched in ST samples, being the top 5 of them (from the more to the less enriched) “metabolic process”, “translation”, “cellular amide metabolic process”, and “tricarboxylic acid cycle”.

4. Discussion

The study of cestode development is challenging, as there are no consolidated methods for their genetic manipulation. RNAi approaches are still in development and so far present low efficiency [39,40]. Moreover, there are virtually no cestode developmental mutants, either naturally or experimentally induced [41]. In this scenario, large scale approaches that aim to identify and quantify gene products (RNA or proteins) are needed. Such methods used in comparative studies between tapeworm life-stages could provide data on differentially expressed genes/proteins possibly involved in cestode development [42]. In this work, a proteomic approach was used to investigate qualitative, quantitative and functional differences in the proteomes of *M. corti* TTs and STs.

The higher number of proteins detected in the ST stage (333) in comparison to TTs (238) could be related to the ST's higher degree of morphological and physiological complexity, which is fully strobilated (proglottized and segmented) and sexually differentiated, in comparison to the mostly undifferentiated TT stage [43,44]. Moreover, the sets of TT and ST differential proteins (including exclusively detected and overrepresented ones), comprehend 58, and 147 proteins, respectively. Overall, these qualitative and quantitative differences between TT and ST samples point out to important differences in molecular and cellular processes between these two development stages.

The *M. corti* genome sequence has become recently available [26], improving RNA and protein identification from transcriptomic and proteomic studies. The RNA profiling of *M. corti* TTs and STs has been recently addressed by our group, which described and compared both the miRNA [23] and the mRNA (Basika et al., manuscript in preparation) repertoires of these two developmental stages. Previous *M. corti* proteomic studies, however, were restricted to the TT stage and to the first 24 h post-induction of strobilation [24,25]. These pioneer *M. corti* proteomic studies identified overall numbers of 87 [24] and 143 [25] *M. corti* proteins, using 2DE-MS and in-solution digestion and LC-MS/MS approaches, respectively. In both cases, the MS instrumentation (QTOF mass spectrometers) was of less resolution and sensitivity than that used here (Orbitrap mass spectrometer). This allowed the identification of 364 unique proteins in the present study, which represented an increment of almost two times in the number of experimentally detected *M. corti* proteins, with 249 being detected the first time.

Among the identified proteins, there were 17 whose genes were annotated as hypothetical ones [26]. Therefore, the presented MS data validated them as genes coding for proteins of so far unknown function. Seven out of these 17 unknown proteins were found as stage-exclusive (1 for TTs, and 6 for STs), and 4 of them have orthologs only among cestodes.

Our data allowed the establishment of a set of *M. corti* proteins without differences in abundance between the TT and ST stages. This non differential set of proteins include many proteins of interest, by their likely involvement in cell regulatory processes, signaling, and proliferation, including 14-3-3 proteins, a mitochondrial ATP synthase, and a dynamin. Out of the 4 detected isoforms of 14-3-3 proteins in our study, 2 were equally represented in TT and ST samples. As conserved regulatory molecules in eukaryotes, they interact

with several other proteins [45], and, in *E. granulosus*, 14-3-3 proteins may be involved in cell signaling and matrix-mediated cell adhesion [46]. The mitochondrial ATP synthase presence in both TT and ST stages was evidenced by the detection of its O subunit in both samples. This enzyme, essential for the ATP-supplying machine in eukaryotic cells [47], and, in the nematode *Caenorhabditis elegans* [48] it has been shown to regulate growth rate and body size. Dynamin, in turn, is a GTPase essential to many cell functions, from endocytosis to organelle division and fusion, and plays a critical role in many physiological functions such as synaptic transmission and muscle contraction [49], and may be involved in development [50]. Interestingly, two FABPs were also found in the *M. corti* set of non-differential proteins, while others of the same family were found as differentially represented in TTs (1 FABP) and STs (3 FABPs). The shared and roughly equally represented FABPs would represent shared lipid-related pathways, while other lipid-related pathways are predominantly expressed in TTs or STs, as discussed below. Overall, our description of the *M. corti* set of non-differential proteins points out to activities and/or functions essential for parasite's survival and development, providing targets for multistage diagnostic/therapeutic approaches.

Functional enrichment analyses of TT and ST differentially abundant proteins highlighted the differences between these two developmental stages. The set of overrepresented functions in TT samples is mostly associated to basic cellular functions, with the enrichment of fewer GO categories (28 functions). On the other hand, the repertory of ST overrepresented proteins in ST is functionally enriched with a greater variety of cellular functions (95 functions). The differential abundance of functional categories between TTs and STs likely reflects the morphological and physiological contrasts between these life cycle stages. The TT stage is less differentiated and its evidenced high metabolic activity would be associated to growth and vegetative development [18,51], essentially limited to asexual reproduction within the intermediate host peritoneal cavity. The ST, on the other hand, is more complex, as a fully strobilated and sexual differentiated stage, which correlates with the overrepresentation of functions associated to protein synthesis and post-translational processing.

The availability of nutrients at the site of parasitism of both TT and ST stages differs significantly, and cestode adaptations to parasitism include extensive reductions in overall metabolic capability and increased ability to absorb nutrients [52,53]. Cestode main energy sources are carbohydrates, which can be catabolized by aerobic or by anaerobic pathways [54]. In TTs, several categories associated with "carbohydrate metabolism" were enriched, which would be in line with findings showing that the *M. corti* larval cellular energy supply is predominantly provided by anaerobic metabolism [55]. TTs and other cestode larvae would uptake glucose from the peritoneal fluid to convert it into lactate when limited amounts of oxygen are available [56]. This would allow the larvae to survive anaerobically for long periods without loss viability. STs, on the other hand, live in the definitive host's small intestine, in an environment richer in energy sources. In line with that, a larger variety of categories associated with energy metabolism (at least four) were enriched in STs, including aerobic metabolism (with enrichment in "cellular respiration", "tricarboxylic acid metabolism" functions). Therefore, our results point out to a fundamental metabolic reprogramming upon the switch of host and the transition between the TT and the ST stage.

Proteins that are differentially abundant between TT and ST samples also point out to different molecular pathways. It is noticeable that the insulin signaling pathway and the related PI3K-Akt signaling pathway, are among those with more protein components represented in TTs. Modulation of these pathways can be mediated by protein phosphorylation/dephosphorylation [57], and, in TTs, such post-translational modification dynamics could be mediated with the participation of the serine/threonine protein phosphatases PP1 and PP2A, which were exclusively detected in the TT stage. In parasitic helminths, previous studies have demonstrated important roles of host insulin or insulin-like peptides of parasite origin in larval development [58,59]. For *M. corti* and *E. multilocularis*, the effects of human insulin in tyrosine-phosphorylation status, glucose content, survival and asexual reproduction rate have been described [57,60]. The possible PP1 and PP2A functions in the regulation of insulin-like signaling pathways and the effects of these pathways in the strobilation of *M. corti* and other cestodes remain to be investigated.

Fatty acid metabolism and glycerolipid metabolism were identified among the pathways more represented in STs. These pathways, likely involved in the metabolism of sterols and fatty acids uptaken from the host, are essential for parasite's survival, as gene losses during cestode evolution made these organisms unable to synthesize fatty acids and cholesterol *de novo* [53,61]. The observed representativity of lipid metabolism-related pathways possibly reflects the preferential use of lipids as carbon sources by STs, which would be associated with the higher energy demand of this more differentiated developmental stage in comparison to TTs.

Ras GTPase superfamily members are regulators that relay intracellular signals [62] and some of these proteins were found as stage-exclusive in our proteomic data. Three were found only in TTs, and one only in STs, suggesting stage-specific roles. In *M. corti*, as in other organisms, Ras-like GTPases may be involved, along with surface receptor tyrosine kinases, in the transduction of insulin or insulin-like signals [57,60,63], and may control parasite's growth and development [64,65]. The Ras-like GTP-binding protein RhoA detected only in the ST stage is a candidate regulator of strobilation, as its *C. elegans* ortholog regulates processes like body morphology, and sexual reproduction [66].

Among the proteins detected exclusively in STs, a Tudor staphylococcal nuclease (TNS) *M. corti* ortholog (TSN-1) is of interest because of its relation with small RNA regulators. As recently shown by our group, some miRNAs are differentially expressed between TTs and STs, and are likely responsible for gene expression regulation during *M. corti* development [23]. TSN, an evolutionarily conserved protein with invariant domain composition, is a subunit of the RNA-induced silencing complex (RISC) [67,68]. In *C. elegans*, *in vivo* studies indicated that TNS-1 is required for the proper function of the let-7 miRNA, essential for the transition from larval stage 4 (L4) to adulthood [69,70]. The let-7 miRNA occurrence in *M. corti* was demonstrated and it was detected as one of the 5 most expressed miRNAs in both TTs and STs [23]. Therefore, it will be interesting to investigate whether the *M. corti* let-7 is under differential regulation by the TNS-1 containing RISC complex.

Some proteins become also of special interest based not only in their possible activities/functions, but also on their higher overrepresentation in terms of FC (> 1), like papilin and

TCP1β. Papilin, overrepresented in TT (FC 2.41), is an extracellular matrix glycoprotein which, in *C. elegans* and *Drosophila*, has been implicated in cell extension and migration processes essential for development [71,72]. In TTs, cell migration is expected to occur during asexual reproduction by fission/budding, and papilin may play some role in this process. The TCP1β, on the other hand, is overrepresented in STs (FC 2.50). It is part of a chaperone complex, which include several other proteins, three of which were also detected in ST samples. It has been shown that the T-complex is essential for efficient chaperone-mediated tubulin biogenesis, and for microtubule cytoskeleton assembly [73]. Such cellular events may be important for the development the new proglottids in STs.

The proteomics results described here provided an extensive list of cestode proteins of interest for both basic and applied studies, not only in *M. corti*, as a model species, but also in cestodes of medical and veterinary importance, like *Echinococcus* spp. and *Taenia* spp. (Table S6). Upon investigation of their spatiotemporal pattern of expression, some of the identified stage-specific proteins, if associated to specific tissues, structures or differentiation processes, are expected to provide a repertoire of molecular markers, very useful and necessary for developmental studies [16]. Moreover, the association of many of the identified proteins to biological processes essential for cestode survival, development and reproduction, make them natural candidates for druggability studies, aiming the development of novel and more efficient drugs against cestodiases and other helminthiases. Finally, upon *in silico* prediction of antigenicity, *Echinococcus* spp. orthologs of at least some of the identified *M. corti* proteins may be of interest as diagnostic antigens, still necessary to improve the immunodiagnosis methods currently used for human echinococcoses [74].

5. Conclusions

The results presented in this work provided a comprehensive profiling of the protein repertories of *M. corti* TTs and the STs. These two developmental stages have remarkable differences in morphology, reproduction, host specificity, and site of infection. Global proteins repertories and the corresponding functional annotations/categorizations of differentially abundant proteins reflected these biological differences between TTs and STs, and provided clues on the cestode strobilation process. Overall, the performed proteomics survey and subsequent comparative analyses highlighted relevant proteins to the development of *M. corti* and other cestodes, with implications for both basic and applied science. The lists of identified proteins are valuable sources for mining targets for functional studies, for the identification of developmental marker molecules, and for the development of novel strategies for diagnosis, control or treatment of cestodiases and other helminthiases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2017.12.022.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the Parasite Genomics Group at the Wellcome Trust Sanger Institute (UK), for providing access to updated *M. corti* annotation data. This work was supported by *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) (AUX-PE-Parasitologia 1278/2011). J.C.L. and G.P.P. are recipients of CAPES M.Sc., and Ph.D. fellowships, respectively.

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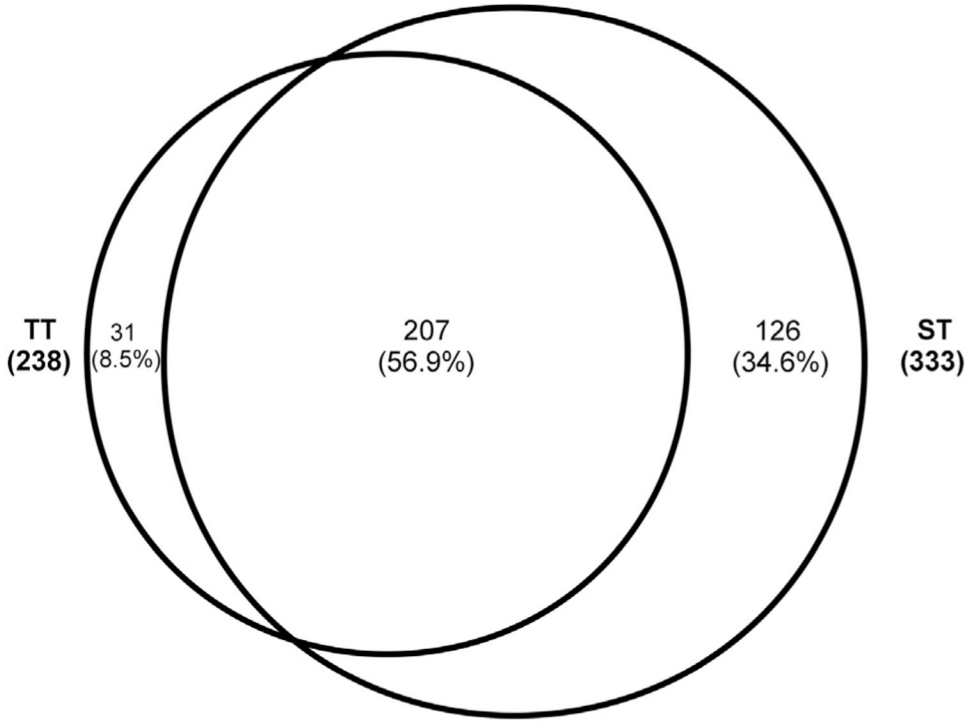

Fig. 1.

Overview of the proteins identified in the TT and ST samples. The numbers and percentages of proteins exclusively detected in each sample or shared between them are indicated in the diagram. The overall numbers of proteins detected in TT and ST samples are indicated in bold. Only proteins identified by at least two peptides and present in at least two biological replicates were considered for analysis.

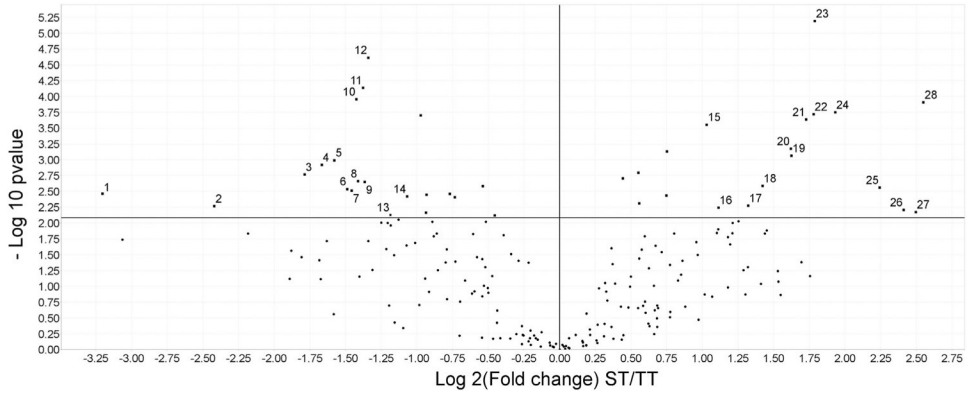

Fig. 2.

Volcano plot of shared TT and ST proteins showing significant differences between samples. Proteins with a p value < .05 and a fold-change (FC) > 1 are represented by black square and identified by their numbering. Differential protein annotations are as follows: (1) lamp; (2) papilin; (3) Prkar2; (4) tubulin alpha-2 chain, (5) innexin family-containing protein, (6) TB2/DP1/HVA22, (7) putative phosphoglucomutase, (8) lysosomal alpha-glucosidase, (9) glycogenin-related, (10) paramyosin, (11) small heat shock protein p36, (12) putative gelsolin, (13) myosin, essential light chain, (14) l-lactate dehydrogenase, (15) pol-related, (16) heat shock protein 60 kDa, (17) dihydrolipoyl dehydrogenase, (18) SDH1, (19) malate dehydrogenase, (20) DLAT, (21) putative 60s ribosomal protein L4, (22) 40S ribosomal protein S7, (23) pyruvate dehydrogenase, (24) 2-oxoglutarate dehydrogenase, (25) sideroflexin 1, (26) T-complex protein 1 subunit beta, (27) NDUFV1; and (28) GCAT KBL. Proteins without significant differences in abundance between samples are shown as black dots.

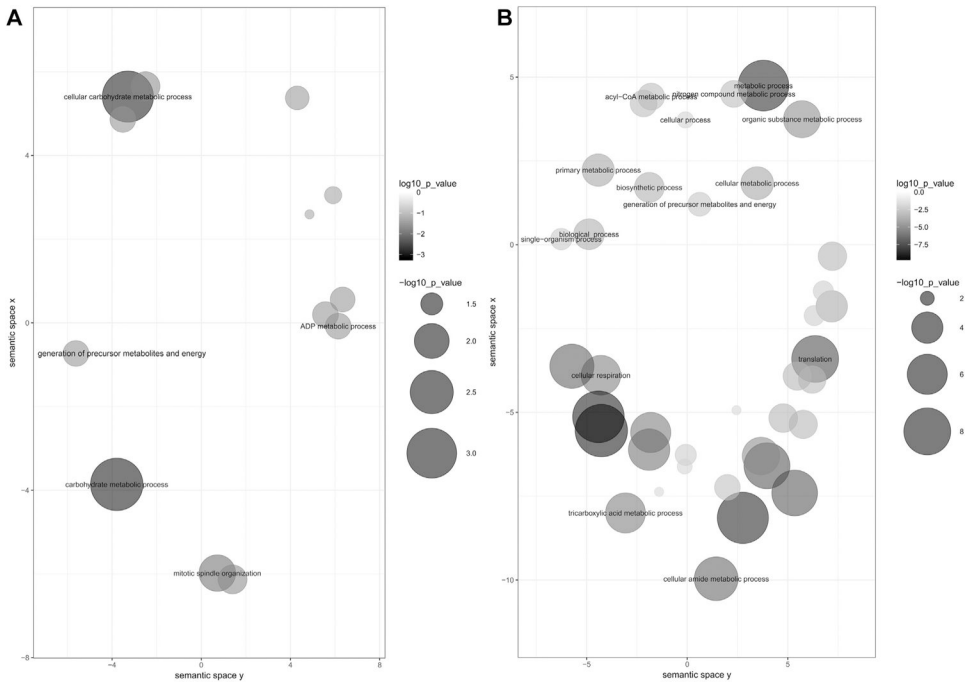

Fig. 3.

Summarized functional classification of differentially abundant proteins identified in the TT and ST samples. Scatterplot view of REVIGO category clusters (summarizing related functional GO-terms) enriched in TTs (A) and STs (B). Only “Biological process” categories are shown. Color and size are proportional to the p-value (larger spheres and darker colors indicate more significant p-values, according to the scale). For each cluster, a representative term of the semantically similar terms there included is shown. Similarity of the GO terms was calculated by SimRel (default allowed similarity = 0.7).

Table 1

Top 10 most represented KEGG pathways in differentially abundant proteins detected in TT or ST samples. The numbers of proteins assigned to each biochemical pathway are indicated. Bi-directional best hit method to assign the orthologs (threshold of BLAST bit scores = 50) were the parameters used. The representative gene data set for eukaryotes plus that of *S. mansoni* were used as references in KAAS mapping.

Tetrathyridia		**Strobilated worm**	
Pathway name	**Number of the represented proteins in TT samples**	**Pathway name**	**Number of the represented proteins in ST samples**
ko01100 Metabolic pathways	12	ko01100 Metabolic pathways	33
ko01110 Biosynthesis of secondary metabolites	7	ko01110 Biosynthesis of secondary metabolites	21
ko01130 Biosynthesis of antibiotics	5	ko03010 Ribosome	17
ko00500 Starch and sucrose metabolism	5	ko01120 Microbial metabolism in diverse environments	17
ko04910 Insulin signaling pathway	5	ko01200 Carbon metabolism	16
ko01120 Microbial metabolism in diverse environments	5	ko00020 Citrate cycle TCA cycle	11
ko04261 Adrenergic signaling in cardiomyocytes	4	ko01130 Biosynthesis of antibiotics	18
ko04151 PI3K-Akt signaling pathway	4	ko00190 Oxidative phosphorylation	7
ko04810 Regulation of actin cytoskeleton	4	ko00970 Aminoacyl-tRNA biosynthesis	6
ko04113 Meiosis	4	ko00620 Pyruvate metabolism	6