



# Adaptation of the secretome of *Echinostoma caproni* may contribute to parasite survival in a Th1 milieu

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Received: 3 November 2017 / Accepted: 10 January 2018 / Published online: 12 February 2018  
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## Abstract

*Echinostoma caproni* (Trematoda: Echinostomatidae) is an intestinal trematode, broadly employed to study the host-dependent mechanisms that govern the evolution of intestinal helminth infections. Resistance against *E. caproni* homologous secondary infections has been reported in mice and appears to be related to the generation of a local Th2 response, whereas Th1 responses promote the development of chronic primary infections. Herein, the ability of *E. caproni* to modulate its secretome according to the host environment is investigated. A two-dimensional differential in gel electrophoresis (2D-DIGE) analysis was performed to elucidate changes in the excretory/secretory products of *E. caproni* adults after primary and secondary infections in mice. A total of 16 protein spots showed significant differences between groups, and 7 of them were successfully identified by mass spectrometry. Adult worms exposed to a primary infection appear to upregulate proteins involved in detoxification (aldo-keto reductase), stress response (GroEL), and enhancement of parasite survival (acetyl-CoA A-acetyltransferase and UTP-glucose-1-phosphate uridylyltransferase). In contrast, any protein was found to be significantly upregulated after secondary infection. Upregulation of such proteins may serve to withstand the hostile Th1 environment generated in primary infections in mice. These results provide new insights into the resistance mechanisms developed by the parasites to ensure their long-term survival.

**Keywords** *Echinostoma caproni* · Trematoda · Secretome modulation · Immune response · Infection resistance · 2D-DIGE

## Introduction

*Echinostoma caproni* (Trematoda: Echinostomatidae) is an intestinal trematode with no tissue phase in the definitive host. After infection, the metacercariae excyst in the duodenum and the juvenile worms migrate to the ileum, where they attach to

the mucosa (Toledo et al. 2009). *E. caproni* has been extensively employed as an experimental model for the study of the factors that determine the establishment of chronic infections or, in contrast, the development of resistance to intestinal helminth infections (Toledo and Fried 2005). To this purpose, two different approaches have been used: (i) comparison of the response to primary *E. caproni* infections in two different host species (mouse and rat) and (ii) comparison of the response to primary and secondary *E. caproni* infections in a single host species (mouse).

Although *E. caproni* has a wide range of definitive hosts, its compatibility differs considerably between rodent species in terms of worm survival and development (Toledo et al. 2004; Toledo and Fried 2005; Muñoz-Antoli et al. 2007). In mice, and other hosts of high compatibility, the infection becomes chronic in relation to a Th1 response, important inflammatory responses, oxidative stress, and marked epithelial injury. In contrast, in hosts of low compatibility (e.g., rat), the worms are rejected within 2–4 weeks post-primary infection in association with the development of a Th2 local phenotype and only mild changes in tissue structure (Muñoz-Antoli et al.

Section Editor: Ramaswamy Kalyanasundaram

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00436-018-5758-1>) contains supplementary material, which is available to authorized users.

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2007, 2014; Toledo et al. 2006; Trelis et al. 2011; Cortés et al. 2015). These facts have allowed studying the factors that determine susceptibility or resistance to infection by comparing the events that occur in mice and rats, respectively, after primary infection.

Although mouse is a permissive host for *E. caproni*, recent studies have shown that a primary infection in CD1 mice induces acquired immunity against subsequent homologous infection manifested by reduced infection rate and worm recovery, and underdevelopment of adult worms (Cortés et al. 2016a; Muñoz-Antoli et al. 2016a). Susceptibility to primary infection relies on a Th1 response, as described above, though resistance to secondary infection is determined by a Th2 response induced by the increase of IL-25 and mild changes in local tissue (Cortés et al. 2016a; Muñoz-Antoli et al. 2016a, b). Because of these characteristics, *E. caproni* mouse model is being used to analyze the mechanisms generating acquired immunity against intestinal helminths and the effector mechanisms leading to worm rejection.

Recently, Cortés et al. (2016b) showed that *E. caproni* is able to change its secretome as an adaptation to different host environments. The secretome of *E. caproni* adult worms showed poor adaptation to the microhabitat generated in the rat intestine, which may facilitate its rapid rejection from this host. In contrast, the excretory/secretory profile of mice-grown worms suggests a better adaptation of the parasite to the Th1-type environment developed in the intestine of this host, thereby enabling its chronic establishment. Herein, we study the changes in the secretome of *E. caproni* in adults collected after primary and secondary infections in mice. Considering the different outcome of each type of infection, the results may aid to the identification of the factors that govern parasite adaptation, as an approach to develop new strategies for the control of helminthic infections. Moreover, the identification of proteins implicated in parasite resistance may help to recognize useful targets for drug and vaccine development.

## Material and methods

### Animals and infection procedures

A total of 28 male, CD1 mice (weighing 30–35 g) were infected by gastric gavage with 75 metacercariae of *E. caproni* and randomly allocated in two groups: 12 mice in group A and 16 mice in group B. The strain of *E. caproni* employed and the infection procedures were described previously (Fujino and Fried 1993). Briefly, encysted metacercariae of *E. caproni* were removed from kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used for infection. At 2 weeks post-primary infection, animals in group A were sacrificed and *E. caproni* adults were collected

from the small intestine. Mice in group B were maintained infected for 4 weeks before treatment with a double dose of 100 mg/kg of praziquantel, orally administrated in alternate days (Cortés et al. 2016a). The elimination of the intestinal infection was confirmed by coprological examination, as previously described (Toledo et al. 2004). Two weeks after antihelminthic treatment, these mice were secondarily infected with 75 metacercariae and maintained for 2 weeks post-secondary infection to collect the worms. The statistical comparison of the percentage of worms recovered in each group was performed by Student's *t* test for independent samples. Animals were maintained under conventional conditions, with food and water ad libitum. This study has been approved by the Ethical Committee of Animal Welfare and Experimentation of the University of Valencia (ref. #A18348501775). Adults from primary and secondary infections were fixed in Bouin's fluid under coverslip pressure, stained with Grenacher's borax carmine, and mounted in Canada balsam.

### Obtaining of excretory/secretory products

To obtain the excretory/secretory products (ESPs), *E. caproni* adults, coming either from primary or secondary infections, were washed with pre-heated RPMI 1640 culture medium (Gibco®, Life Technologies) and maintained at concentrations of 40 worms/ml for 12 h at 37 °C in RPMI containing 100 U penicillin, 100 mg/ml streptomycin, and cComplete mini EDTA-free protease inhibitor cocktail (Roche). After incubation, the worms were still alive. The medium was collected and centrifuged at 15,000×*g* for 30 min at 4 °C. After centrifugation, the supernatant was collected and protein concentration was measured using Bio-Rad protein assay. To increase the biological significance and avoid erroneous conclusions due to individual variations, biological replicates were prepared as described previously (Cortés et al. 2016b), incubating a total of 120 worms recovered from different hosts in each replicate. Four biological replicates were performed for each experimental group, i.e., primary and secondary infection.

### Protein labeling and two-dimensional differential in gel electrophoresis

A total amount of 50 µg of protein from each biological replicate was cleaned and precipitated with 2D Clean-up Kit (GE Healthcare); pellets were resuspended in 18 µl of a proper buffer (25 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5), and proteins were fluorescently tagged with CyDye DIGE Fluor minimal dyes (GE Healthcare), following the manufacturer's instructions. One microliter of dye (400 pmol) was added to each sample and maintained on ice for 30 min in the dark. The reaction was stopped by adding 1 µl of 10 mM lysine. To minimize any dye-specific labeling artifacts, two biological replicates of each experimental group were labeled with Cy3

and the other two with Cy5. The internal standard, prepared by mixing the same amount of protein of each sample in the experiment, was always labeled with Cy2.

The ESPs were compared across four 2D-DIGE gels to investigate differences in protein production and/or release that may be associated with the establishment of primary and secondary infections. Each of the four pairs of Cy3- and Cy5-labeled biological replicates (50 µg of protein each) were combined with a 50 µg aliquot of the Cy2-labeled internal standard. The mixtures were then separated in the first dimension, i.e., isoelectric focusing, and the second dimension. The IPG strips (24 cm, nonlinear pH 3–11) were rehydrated overnight with rehydration buffer (8 M urea, 4% CHAPS, 1% ampholytes, and 12 µl/ml of DeStreak™), and the labeled samples were then applied to the strips by anodic cup loading, after the addition of DTT and ampholytes up to a final concentration of 65 mM and 1%, respectively. The isoelectric focusing was carried out at 20 °C in the Ettan IPGphor 3 System (GE Healthcare) as follows: (i) 300 V for 4 h, (ii) gradient to 1000 V for 6 h, (iii) gradient to 8000 V for 3 h, and (iv) 8000 V up to 32,000 Vh. Prior to the second dimension, the strips were equilibrated in two steps, 15 min each, in equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, and 2% SDS) containing either 2% DTT or 2.5% iodoacetamide, respectively. The separation of proteins in the second dimension was performed in an Ettan DALTsix system (GE Healthcare) using 12.5% polyacrylamide gels. Electrophoresis was run at 1 W/gel for 1 h followed by 5 h, approximately, at 15 W/gel.

### Imaging and comparison of 2D protein profiles

Gels were scanned in a Typhoon™ 9400 Variable Mode Imager (GE Healthcare) at appropriate wavelengths for each fluorophore: Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm), and at 50-µm resolution. The nonessential information was removed using ImageQuant Tools software, and SameSpots software (TotalLab) was employed for image analysis. Prior to analysis, images were quality checked to identify the internal standard and select a reference image. The 12 gel images were then aligned to the representative internal standard to eliminate the positional variation introduced during gel electrophoresis and guarantee 100% spot matching across all images. Spot detection was performed with the in-built software routines and manually supervised to ensure that each spot was well defined. Minimal editing was needed to exclude artifacts, overlapping spots, or single spots that were recognized as two. The edited datasets were then transferred to the SameSpots statistical package for analysis. Protein spots showing statistically significant differences in the average normalized volumes between the ESPs of parasites recovered from primary and secondary infections were selected using Student's *t* test ( $p < 0.05$ ).

### Protein identification by LC-MS/MS and database search

Differential spots were manually excised from the gel, washed twice with double-distilled water, and digested with sequencing grade trypsin (Promega). For liquid chromatography and tandem mass spectrometry (LC-MS/MS), digested samples were diluted in 12 µl of 5% formic acid and 6 µl of the resulting suspension were injected onto a 50 mm × 300 µm C18 trap column (Agilent Technologies) using a Shimadzu Prominane Nano HPLC. Samples were desalted on the trap column for 5 min using 0.1% formic acid (aq) at 30 µl/min. Peptides were then eluted onto an analytical nano HPLC column (150 mm × 75 µm 300SBC18, 3.5 µm, Agilent Technologies) at a flow rate of 300 nL/min and separated using a 35-min gradient of 1–40% buffer B followed by a steeper gradient from 40 to 80% buffer B in 5 min. Buffer B contained 90/10 acetonitrile/0.1% formic acid, and buffer A consisted of 0.1% formic acid (aq). The column eluates were subsequently ionized using a 5500 QTRAP system (AB Sciex) operated in an information dependent acquisition (IDA) mode. Full-scan TOFMS data was acquired over the mass range 350–1400, and for product ion MS/MS 80–1400 *m/z*, ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, MS/MS spectra of the resultant 20 most intense ions. Database search was performed using MASCOT 2.5 (Matrix-Science) search engine on the *E. caproni* genome database, available online at [http://parasite.wormbase.org/Echinostoma\\_caproni\\_prjeb1207/Info/Index/](http://parasite.wormbase.org/Echinostoma_caproni_prjeb1207/Info/Index/). Searches were done with tryptic specificity, allowing one missed cleavage and a tolerance in mass measurement of 100 ppm in MS mode and 0.6 Da for MS/MS ions. Carbamidomethylation of Cys was used as fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications. Only proteins identified with two or more significant peptides were taken into account. BLASTp was performed against NCBI nr protein database with taxonomy set in Trematoda.

### Immunohistochemical localization of GroEL

Two-week-old *E. caproni* from primarily infected mice were fixed in Karnovsky's fixative (0.5 M glutaraldehyde, 2.5 M formaldehyde), washed in phosphate buffer 0.1 M, pH 7.2, and post-fixed in 2% osmium tetroxide in phosphate buffer prior to inclusion in LR-white resin. After several washes in water, parasites were sequentially dehydrated in 30, 50, 70, and 96% ethanol, and incubated, for 2 h each, in 33% LR-white resin in 96% ethanol, 66% LR-white resin in 96% ethanol, 66% LR-white resin in 100% ethanol, and 100% LR-white resin in 100% ethanol. Parasite sections were blocked for unspecific unions with a solution of 5% BSA in PBS

containing 0.3% Triton X-100 (PBS-TX). Primary antibody, rabbit anti-human GroEL/HSP60 (119-12691, RayBiotech), diluted 1/20 in PBS-TX containing mouse serum 10%, was applied overnight at 4 °C under continuous agitation. After washing, sections were incubated with secondary antibody, donkey anti-rabbit IgG (RPN2124, GE Healthcare) diluted 1/100 in PBS-TX, for 2 h at room temperature. Sections were washed in PBS and revealed with a solution of 0.05% diaminobenzidine (Sigma-Aldrich) in water containing 0.025% H<sub>2</sub>O<sub>2</sub>. Negative controls were processed as samples, without incubation with primary antibody.

### Total RNA extraction and RT-PCR real time

Total RNA was isolated from full-thickness sections of the ileum of uninfected mice and 2 weeks after primary or secondary infection using Real Total RNA Spin Plus Kit (Durviz), and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For quantitative PCR, 9 µl of the product of reverse transcription, diluted 1/10 in sterile water, was added to 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystem) and 1 µl of the pertinent TaqMan® Gene Expression Assay: Mm99999071\_m1 for IFN-γ, Mm00445259\_m1 for IL-4, Mm99999190\_m1 for IL-13, and Mm01205647\_g1 for β-actin, which was used as a housekeeping gene to normalize for differences in the efficiency of sample extraction and/or cDNA synthesis. Reactions were performed on the Abi Prism 7000 (Applied Biosystems), with the following thermal cycler conditions: an initial step of 10 min at 95 °C followed by 40 cycles of 15 s denaturation at 95 °C and 1 min of anneal/extension at 60 °C each. Both samples and controls were analyzed in triplicate. The threshold cycle (Ct) was calculated for the genes of interest and the housekeeping in each sample and negative control, and a comparative quantification method ( $2^{-\Delta\Delta C_t}$ ) was applied to evaluate the effect of the infection on gene expression (Livak and Schmittgen 2001). The method is based on the fact that the difference in threshold cycles ( $\Delta C_t$ ) between the gene of interest and the housekeeping is proportional to the relative expression of the gene of interest. The fold change in the target genes was normalized to β-actin and relativized to the expression in uninfected animals to get a relative quantification of the expression levels (Klein 2002).

### Statistical analysis

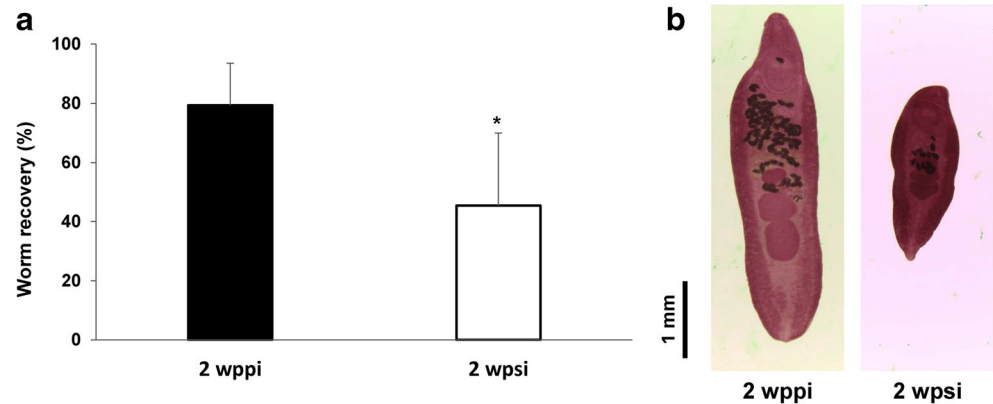
Comparison of cytokine expression levels between infected mice and basal values prior to each type of infection and levels was performed by unpaired t test.  $p < 0.05$  was considered as significant. Prior to analyses, data were log transformed to achieve normality.

## Results and discussion

In order to study the ability of *E. caproni* to adapt its proteome to different host milieus and its consequences in the parasite survival, herein the secretome of adult worms exposed either to primary and homologous secondary infections in CD1 mice has been analyzed. Primary infection is associated with the development of chronic infections, with 100% rate of infection and high worm recoveries, concomitantly with local Th1-type inflammatory responses and oxidative stress. In contrast, secondary infections are characterized by the development of partial resistance, with significantly lower infection rates and worm recoveries, and the underdevelopment of adult worms associated with a Th2 response and mild tissue damage (Muñoz-Antolí et al. 2007; Cortés et al. 2016a; Muñoz-Antolí 2016a). The present study has demonstrated the differential effect of Th1 and Th2 responses on the secretome of *E. caproni* adult worms. Our results indicate that Th1 responses in primary *E. caproni* infections induce upregulation of several proteins, which may facilitate the chronic establishment of the parasite.

According to previous studies (Cortés et al. 2016a; Muñoz-Antolí et al. 2016a, b), the number of worms recovered per mouse decreased significantly in secondary infections compared with primary infections ( $p = 0.031$ ) (Fig. 1). Moreover, collected worms were phenotypically different. *E. caproni* from secondarily infected mice were smaller and underdeveloped in comparison from worms from primary infections (Fig. 1), though other signs of damage were not appreciated in worms belonging to any group. These changes have been previously attributed to the different immune response elicited against primary and secondary infections, and they are not related to exposure to praziquantel (Cortés et al. 2016a; Muñoz-Antolí 2016b). Herein, gene expression levels of prototypical Th1 and Th2 cytokines (IFN-γ and IL-4, IL-13, respectively) were measured in the intestine of primarily and secondarily infected mice (Fig. 2). As previously described (Trelis et al. 2011; Muñoz-Antolí et al. 2016b), primary infection induces significant upregulation of IFN-γ mRNA, while resistance to secondary infection is associated with augmented levels of local IL-4 and IL-13 (Fig. 2). *E. caproni* adults are known to modulate their secretome in response to the host environment, and this can be translated into a better adaptation and survival into some ambiances, while not others (Cortés et al. 2016b). In order to study the influence of the distinct immunological milieu between primary and secondary infections on parasite protein secretion, the ESPs of each type of worms were subjected to 2D-DIGE to identify differentially produced and/or secreted proteins. A total of 602 protein spots matched through the four gels included in the analysis, and 16 of them showed significant statistical differences between groups ( $p < 0.05$ ) (Fig. 3 and Online Resource 1). All the differential spots displayed higher protein abundance in the

**Fig. 1** **a** Percentage of worms recovered from the intestine of infected mice at 2 weeks post-primary and secondary infections (wppi and wpsi, respectively). **b** Representative specimens of *Echinostoma caproni* adults recovered from each experimental group

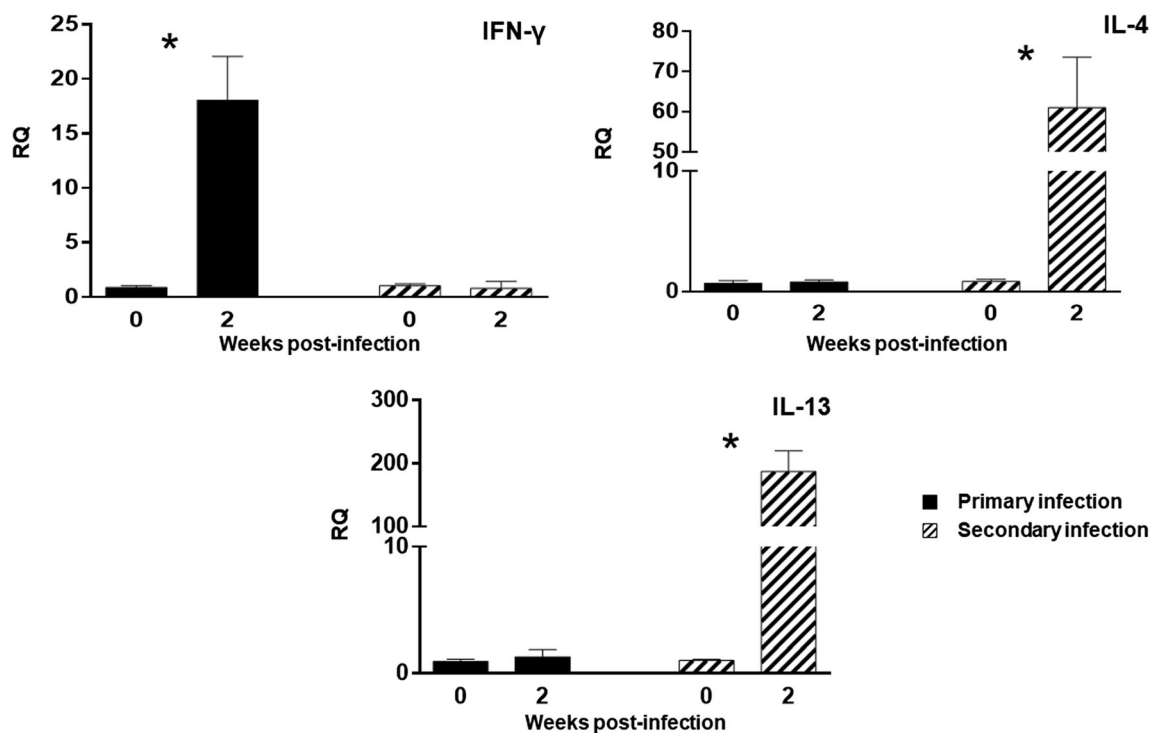


ESPs of worms collected from primarily infected mice, and fold differences (F) between the average normalized volumes for each group ranged between 1.4 and 2.7. The details of the computational comparison are shown in Online Resource 2.

A total of seven differential protein spots were successfully removed from gels and identified by mass spectrometry and database search (Table 1). Among the identified proteins, there were metabolic enzymes, such as UTP-glucose-1-phosphate uridylyltransferase (UTPGT) (F: 1.4), acetyl-CoA C-acetyltransferase (ACAT) (F: 1.7), and triose phosphate isomerase (TPI) (F: 1.4); an antioxidant enzyme, belonging to the superfamily of aldo-keto reductases (AKRs) (F: 1.8);

and the chaperonin GroEL (F: 2.4). Moreover, the structural protein actin was identified in two spots (F: 1.6 and 1.9, respectively).

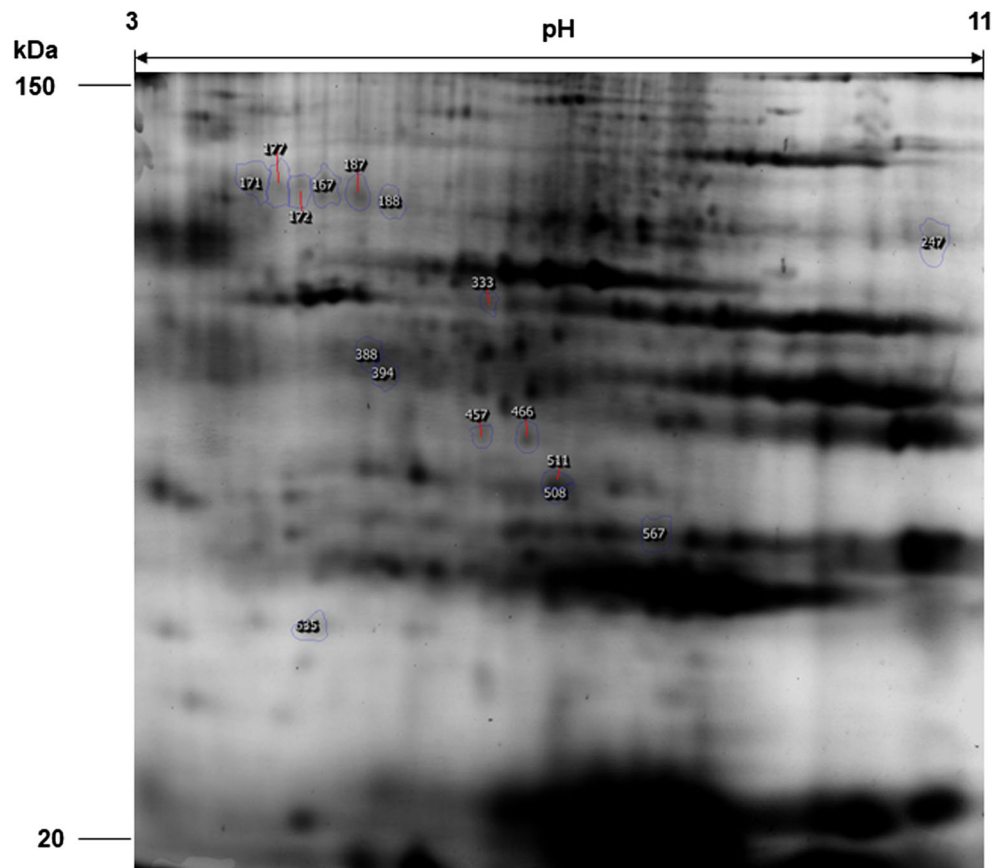
Although the number of differentially secreted proteins was not as high as initially expected, in view of the differences in worm recovery and size (Fig. 1), proteomic results are consistent with the environment generated in each type of infection. Th1 primary response induced upregulation of several proteins (ACAT, AKR, UTPGT, and GroEL) related to the response against oxidative stress and the enhancement of parasite survival. This fact may contribute to the long survival of *E. caproni*, in spite of the hostile environmental conditions



**Fig. 2** Expression of cytokine mRNA in the intestinal tissue of CD1 mice in primary and secondary infections with *Echinostoma caproni*. In primary infections, time point 0 reflects the values in naïve animals and in secondary infections represents the basal values of cytokine expression at the moment of infection in mice cured of a primary infection. The

relative quantities (RQ) of cytokine genes are shown after normalization with  $\beta$ -actin and standardization of the relative amount against day 0 sample. Vertical bars represent the standard deviation. Asterisk indicates significant differences with respect to respective controls at time 0 in each type of infection ( $p < 0.001$ )

**Fig. 3** Representative 2D-DIGE image of *Echinostoma caproni* excretory/secretory products. Differentially abundant spots are indicated (Table 1 and Online Resource 2)



induced by that Th1 response against primary infections in CD1 mice.

ACAT is a member of the thiolase superfamily, which includes a number of enzymes that share a common evolutionary origin and high degree of sequence similarity (Iguar et al. 1992; Bun-Ya et al. 1997). Eukaryotic thiolases have key roles in many biochemical pathways, such as  $\beta$ -oxidation of fatty acids, as well as in several biosynthetic routes. Moreover, thiolases have been shown to be upregulated under stress conditions, playing a crucial role in delaying aging of helminths and extending lifespan. Loss-of-function mutations in the ketoacyl thiolase gene (*kat-1*) of the model nematode *Caenorhabditis elegans* resulted in premature aging and a shortened lifespan. Under stress conditions, metabolism switches from the use of glucose to fatty acid oxidation by thiolases, providing further resistance and enhancing worm survival (Berdichevsky et al. 2010). Furthermore, it has been shown that thiolases increase the survival of organisms by interfering with apoptosis. Cao et al. (2008) showed that the thiolase acetyl-CoA A-acetyltransferase attenuated the proapoptotic effect of BNIP3 in human cells. Genes encoding for enzymes involved in fatty acid metabolism are intact and expressed in several trematode species, such as *Clonorchis sinensis*, while not in others as *Schistosoma japonicum* (Wang et al. 2008; Zhou et al. 2009), though their roles in

the course of the infection are not well known. Lin et al. (Lin et al. 2015) studied in detail the role of acetyl-CoA thiolase of *C. sinensis* and showed that environmental conditions modulate the production of this protein. Under stress conditions, this thiolase became upregulated, increasing parasite survival. This upregulation served to enhance the  $\beta$ -oxidation of fatty acids as energy source to survive longer. Our results support that *E. caproni* ACAT is modulated by the host immune response and its overregulation may serve to extend parasite survival in primary infections. Under the stress milieu generated by Th1 primary response, ACAT is upregulated concomitantly with the development of *E. caproni* chronic infections. In contrast, early expulsion and underdevelopment of adult worms in secondary infections are associated with significantly lower production of this enzyme.

AKRs comprise a group of structurally related proteins of a common ancestry that have been found in a wide range of phyla, including both prokaryotes and eukaryotes. AKRs catalyze redox transformation in a number of cellular processes that involve biosynthesis, intermediary metabolism, and detoxification. Substrates of AKRs include glucose, steroids, glycosylation end-products, lipid peroxidation products, and environmental pollutants (Barski et al. 2008; Mindnich and Penning 2009). AKRs have been previously reported in

**Table 1** Identification details of differentially abundant protein spots

Identification details			BLASTp details <sup>f</sup>							
Spot <sup>a</sup>	Accession number <sup>b</sup>	Protein	Species (Acc. <sup>c</sup> )	MASCOT score	Matches <sup>d</sup>	Peptides <sup>e</sup>	<i>E</i> value	Total score	Query cov (%)	Ident (%)
167	ECPE_0001629101-mRNA-1	Chaperonin GroEL	<i>Clonorchis sinensis</i> (GAA56729)	477	17	15	0	1259	97	89
247	ECPE_0001457601-mRNA-1	UTP-glucose-1-phosphate uridylyltransferase	<i>C. sinensis</i> (GAA27364)	127	6	5	$1 \times 10^{-84}$	259	69	66
388	ECPE_0000454001-mRNA-1	Actin-2	<i>Schistosoma haematobium</i> (XP_012798726)	368	10	10	0	784	91	99
394	ECPE_0000454001-mRNA-1	Actin-2	<i>S. haematobium</i> (XP_012798726)	337	6	6	0	784	91	99
466	ECPE_0000877001-mRNA-1	Aldo-keto reductase family 1, member B4	<i>Schistosoma japonicum</i> (CAX77276)	101	4	3	$4 \times 10^{-77}$	235	81	58
508	ECPE_0000982101-mRNA-1	Acetyl-CoA C-acetyltransferase	<i>C. sinensis</i> (GAA52468)	84	2	2	$2 \times 10^{-96}$	287	65	72
567	ECPE_0000823201-mRNA-1	Triose phosphate isomerase	<i>Fasciola hepatica</i> (AGJ83762)	408	10	9	$1 \times 10^{-107}$	310	81	84

<sup>a</sup> Spot reference number<sup>b</sup> Accession number in the *Echinostoma caproni* genome database<sup>c</sup> Accession number in the protein database of NCBI<sup>d</sup> Number of significant matches<sup>e</sup> Number of significant peptides<sup>f</sup> Details of protein blast of translated genome annotations against the NCBI protein database

*E. caproni*, and they seem to be secreted via exosomes (Guillou et al. 2007; Marcilla et al. 2012; Cortés et al. 2016b). Cortés et al. (2016b) showed that the secretion of these enzymes can be modulated by environmental conditions. After a primary infection, production of *E. caproni* AKR was greater in rats than in mice, probably in relation to host-dependent factors. Herein, we have demonstrated that using the same host species, this protein is overproduced in an ambience of Th1 response. There is increasing recognition of the role of AKRs in preventing toxicity, and several studies have demonstrated that they are more active under stress conditions, particularly in oxidative defense, promoting cellular survival (Chang and Petrash 2008; Farahyar et al. 2013).

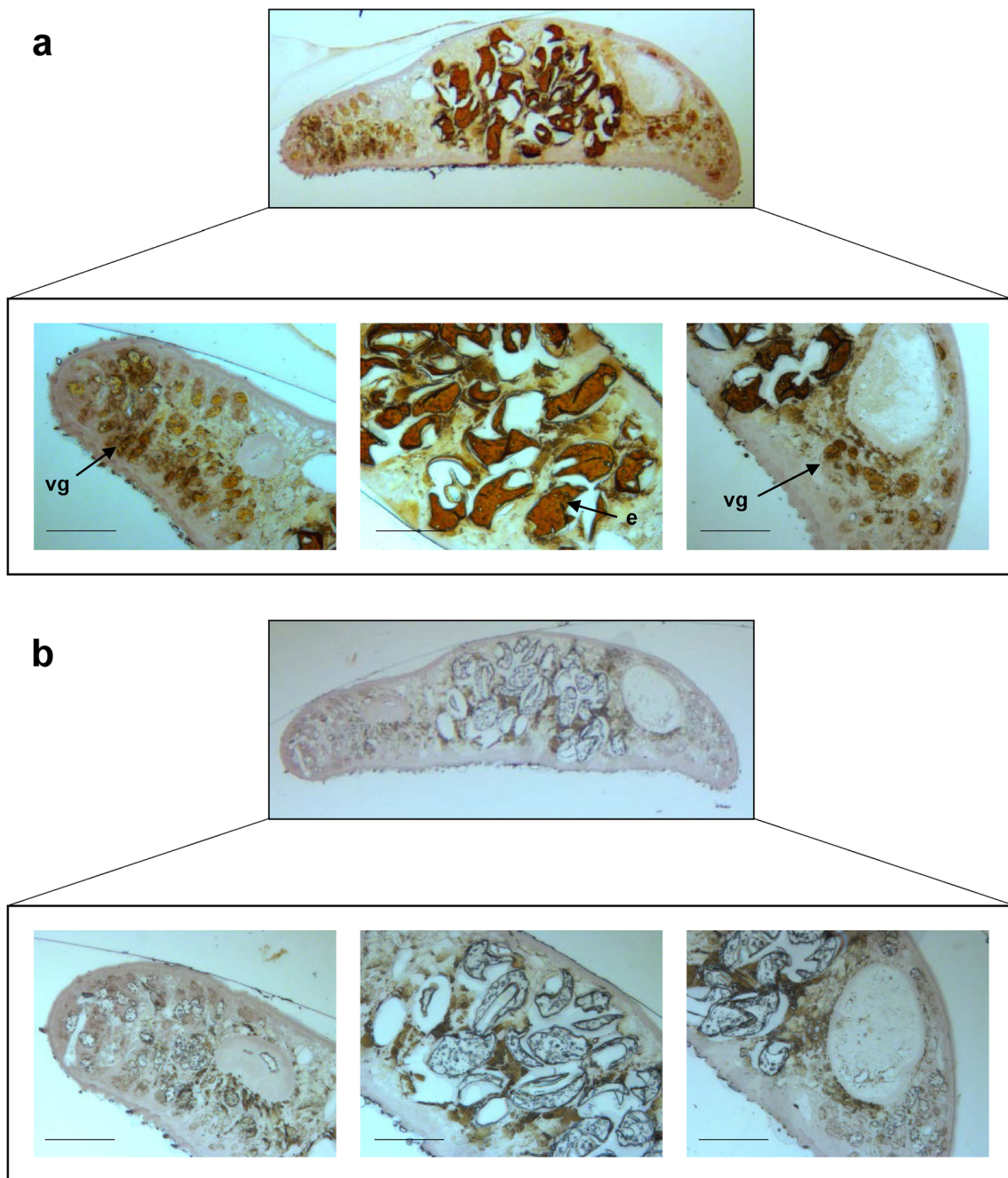
GroEL is a stress-related protein, belonging to the HSP60 family of chaperones. In eukaryotes, GroEL and li-like chaperonin GroES are nearly identical, both structurally and functionally, to HSP60 (Zeilstra-Ryalls et al. 1991; Horwich et al. 2007). GroEL assists in correct folding and assembly of proteins to avoid denaturation and also serves to prevent protein denaturation and reactivate partially denatured proteins under stress conditions. Several toxics and stressing conditions have been shown to increase transcription of GroEL. Oxidative stress is one of the most relevant features upregulating GroEL production, playing an important role for adaptation to adverse (Dosanjh et al. 2005; Sharma et al. 2016). Moreover, GroEL has been found secreted in membrane fractions under stress conditions, suggesting a role in the attachment process of intestinal pathogens (Hennequin et al. 2001). In order to check if *E. caproni* GroEL is involved in mucosal adherence, immunolocalization was performed on parasite sections. Contrary to our expectations, GroEL did not appear accumulated in the tegument nor the external surface of adult worms, but it was localized in vitelline glands and eggs (Fig. 4), suggesting that it may have a role in proper egg formation and maturation, to ensure parasite transmission. According to this result, higher secretion of GroEL after primary infection may be explained by the greater development of worms. Muñoz-Antolí et al. (2016a) showed that adults from primary infections are more developed than those from secondary infections, with higher metric values for different variables, including ovary and testis. Differences in worm growth are confirmed herein (Fig. 1), and different amount of GroEL in ESPs may be indicative of greater gravidity of worms in primary infections. The presence of stress-related proteins in eggs and vitelline glands is a common fact in helminths. GroEL is named as heat shock protein (HSP) 60 in many species. HSP-60 is constitutively and extensively expressed in *Schistosoma japonicum* eggs, and it has been shown to have immunomodulatory properties (Zhou et al. 2015a). Moreover, the related protein HSP-70 is one of the most abundant proteins in the egg secretome of *Schistosoma mansoni* (Cass et al. 2007) and has been previously localized in *E. caproni* eggs (Higón et al. 2008). The absence of GroEL

in the tegument is not surprising since it has been shown that that there is no direct relationship between the amounts of this kind of chaperones in tegument and that present in excretory/secretory products (Higón et al. 2008).

Another protein associated with virulence and pathogen survival that have been found upregulated in *E. caproni* primary infections is UTPGT (alternatively named glucose pyrophosphorylase). UTPGT is involved in carbohydrate metabolism, specifically in glycogenesis, synthesizing UDP-glucose from glucose-1-phosphate and uridine triphosphate. Several studies have identified a number of genes encoding proteins involved in carbohydrate metabolism to be of importance for pathogen survival (Klein et al. 2012). Among these genes, that encoding UTPGT (*galU*) appeared to play a major role (Klein et al. 2012). Moreover, it has been shown that *galU* gene and its product are essential for the success of infections. Priebe et al. (2004) showed that blocking of the *galU* gene in *Pseudomonas aeruginosa* inhibited its ability to infect the cornea of mice and the systemic spread of the bacteria after lung infection. Similarly, *galU* gene has been also found to be critical for pathogenesis and survival of other bacteria (Sandlin et al. 1995; Chang et al. 1996; Rioux et al. 1999; Nesper et al. 2001; Kohler et al. 2002). In the protozoan *Leishmania major*, depletion of this gene resulted in altered expression of lipophosphoglycan and reduced growth and virulence of the parasite (Damerow et al. 2015). In our study, we have observed that fewer production of UTPGT occurs concomitantly with early rejection of *E. caproni* adult worms in secondary infections in mice. Although further studies are required, our results suggest that this protein may be involved in *E. caproni* survival in its definitive host.

The potential role of upregulation of *E. caproni* TPI in primary infections in mice is difficult to ascertain. TPI has been previously reported in ESPs of *E. caproni*, and it was found to be secreted via exosomes (Marcilla et al. 2012). Moreover, this protein has been also reported in several trematodes, such as *Opisthorchis viverrini*, *C. sinensis*, *Fasciola hepatica*, or *Schistosoma* spp. (Saijuntha et al. 2009; Zinsser et al. 2013a, b; Dai et al. 2014; Zhou et al. 2015b). This protein is a key regulatory enzyme of glycolysis and glycogenesis, playing an essential role in the metabolism and development of most organisms (Knowles 1991). TPI catalyzes the conversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Then, D-glyceraldehyde 3-phosphate is further processed in the glycolytic pathway to pyruvate, permitting production of ATP and NADH. Without this reaction, there would be no production of ATP through the glycolytic pathway. Furthermore, TPI participates in other biological functions related to the development of trematodes including energy and metabolite generation, reproduction, and processing of metabolites and nutrient factors (Zhou et al. 2015b). In primary *E. caproni* infections in mice, higher expression of TPI may serve as an energy provider for adult worms,





**Fig. 4** **a** Localization of GroEL by immunohistochemistry on worms at 2 weeks post-primary infection. **b** Negative control. vg vitelline glands, e eggs

promoting the survival of the parasite in an adverse milieu. Zhou et al. (2015b) suggested that *C. sinensis* TPI is essential in the first steps of the infection, to invade the definitive host and to the transformation of juvenile into adult worms. However, the role of TPI should be studied further. It is known that oxidative stress induces redirection of carbohydrate flux, to the generation NADH at the expense of the pentose phosphate pathway (Godon et al. 1998). This makes difficult to explain the upregulation of TPI in primary infections in mice, though it could be a mechanism to compensate repression of glycolysis induced by oxidative stress.

Herein, we have shown that the local immune response and the alterations mediated by this response influence the secretome of *E. caproni* and this has implications for parasite survival. Adult worms exposed to a primary infection in mice upregulate a number of proteins involved in detoxification processes, response to stress, and enhancement of parasite survival. This may serve to withstand the Th1 environment, with inflammatory response and oxidative stress, generated as a consequence of a primary infection, resulting in the development of chronic infections. In contrast, Th2 environment in secondary infections did not induce significant upregulation

of any protein in the secretome of *E. caproni*, coinciding with the early rejection of worms. This confirms that *E. caproni* appears to be better adapted to grow in a Th1 type than in a Th2 environment, which may explain, at least in part, the course of *E. caproni* infection in each milieu.

**Acknowledgements** This work was supported by Ministerio de Economía y Competitividad (Madrid, Spain) (grant number BFU2016-75639-P); Conselleria d'Educació, Generalitat Valenciana (Valencia, Spain) (grant number PROMETEO2014-083 Fase II), and from Ministerio de Sanidad y Consumo (Madrid, Spain) (No. RD12/0018/0013, Red de Investigación Cooperativa en Enfermedades Tropicales (RICET), IV National Program of I+D+I 2008-2011, ISCIII—Subdirección General de Redes y Centros de Investigación Cooperativa and FEDER).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Statement on welfare of animals** All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

**Statement on human rights** This article does not contain any studies with human participants performed by none of the authors.

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