UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

# Análise da expressão gênica durante a indução ao desenvolvimento estrobilar em *Echinococcus granulosus*

Tese de Doutorado

João Antonio Debarba

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# Análise da expressão gênica durante a indução ao desenvolvimento estrobilar em *Echinococcus granulosus*

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, como requisito parcial para a obtenção do grau de Doutor em Ciências

João Antonio Debarba

Orientador: Dr. Arnaldo Zaha
Coorientadora: Dra. Karina M. Monteiro

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**Capítulo I**



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## Resumo

*Echinococcus granulosus* é um parasito cestódeo e o agente etiológico da hidatidose cística. O desenvolvimento dos vermes adultos a partir dos protoscoleces é, provavelmente, desencadeada por diferentes estímulos que são impostos pelos hospedeiros, os quais provocam mudanças moleculares e morfológicas no parasito. No entanto, os mecanismos responsáveis por essas variações não estão totalmente esclarecidos. Para encontrar genes e proteínas possivelmente envolvidos com as alterações fenotípicas observadas durante o desenvolvimento estrobilar de *E. granulosus*, relatamos aqui o perfil transcritômico e a detecção de proteínas recém-sintetizadas após a indução *in vitro* de protoescólices ao desenvolvimento estrobilar. Os protoescólices foram tratados com pepsina e mantidos em meio bifásico contendo taurocolato para induzir ao desenvolvimento estrobilar. Azidohomoalanina, aminoácido análogo de metionina, foi adicionado para a incorporação metabólica nas proteínas recém-sintetizadas, que foram visualizadas por microscopia confocal e identificadas por espectrometria de massas. Paralelamente, o RNA total foi isolado utilizando o reagente TRIzol e as bibliotecas geradas foram sequenciadas na plataforma Illumina MiSeq. Nós identificamos 23 proteínas detectadas exclusivamente na presença de estímulos para o desenvolvimento estrobilar (SSD) e 28 na ausência desses estímulos (NSD). Também encontramos 75 proteínas diferencialmente expressas entre as duas condições (34 em SSD e 41 em NSD). Na análise dos dados de RNA-seq, 818 genes foram identificados como diferencialmente expressos pelo software GFOLD. De forma geral, genes e proteínas com funções moleculares de transdução de sinal, metabolismo e modificações de proteínas foram observadas com a progressão do desenvolvimento estrobilar. Assim, este estudo fornece informações sobre genes e proteínas que podem ter um papel chave para o desenvolvimento do parasito, além de serem alvo de estudos futuros.

## Abstract

*Echinococcus granulosus* is a cestode parasite and the etiological agent of the cystic hydatid disease. The development of the adult worms from protoscoleces is probably triggered by different stimuli imposed by the hosts, which lead to molecular and morphological changes. However, the mechanisms underlying these variations remain largely unclear. In order to find genes and proteins possibly involved with the phenotypic changes during the strobilar development, we reported here the transcriptomic profile and the detection of newly synthesized proteins after protoscoleces *in vitro* induction to strobilar development. Protoscoleces were treated with pepsin and maintained in biphasic medium containing taurocholate to induce the strobilar development. The methionine analogue azidohomoalanine was added for metabolic incorporation in the newly synthesized proteins that were visualized by confocal microscopy and identified by mass spectrometry. In parallel, total RNA from parasite material was isolated using TRIzol reagent and the generated libraries were sequenced on Illumina MiSeq platform. We identified 23 proteins present only after stimuli for strobilar development (SSD) and 28 in absence of these stimuli (NSD). We also found 75 differentially expressed proteins (34 SSD and 41 NSD). In the RNA-seq analysis, 818 differentially expressed genes were identified by the GFOLD software. Gene transcripts and proteins with molecular functions of signal transduction, metabolism and protein modifications were observed with progression of development. This study provides insights on searching for the key genes and proteins that may be important for the correct parasite development and be target for further studies.

# 1. Introdução

## 1.1 O gênero *Echinococccus*

*Echinococcus* spp. são cestódeos endoparasitos pertencentes à família Taeniidae, um importante grupo causador de zoonoses em todo o mundo. A taxonomia do gênero é bastante controversa devido à escassez de características morfológicas no verme adulto e à capacidade do metacestódeo de se desenvolver em uma ampla variedade de hospedeiros (KNAPP et al., 2011; NAKAO et al., 2013).

Nos anos 1980, as diversas espécies e subespécies descritas até então foram revistas e organizadas em quatro espécies distintas: *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus oligarthrus* e *Echinococcus vogeli* (KUMARATILAKE; THOMPSON, 1982; THOMPSON; MCMANUS, 2002). Entretanto, no caso de *E. granulosus*, essa classificação agrupava organismos com diferentes morfologias, especificidade ao hospedeiro, parâmetros bioquímicos, biologia do desenvolvimento e distribuição geográfica. Tais diferenças desencadearam no surgimento de um sistema informal de subdivisão das espécies em linhagens, como *sheep*, *horse* e *cattle strain* (ROMIG; EBI; WASSERMANN, 2015).

Com o uso de técnicas de biologia molecular, foram identificados polimorfismos em genes mitocondriais entre as diferentes linhagens. Os polimorfismos foram correlacionados com as linhagens descritas até então e levaram a uma nomenclatura baseada em genótipos (G1 a G10) (BOWLES; BLAIR; MCMANUS, 1992; BOWLES; MCMANUS, 1993). Nos últimos anos, análises filogenéticas permitiram classificar a maioria dos genótipos como novas espécies: *E. granulosus sensu stricto* (genótipos G1/G2/G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), *Echinococcus canadensis* (G6/G7/G8/G10), que se somaram às demais espécies do gênero, *E. multilocularis*, *E. vogeli*, *E. oligarthrus*, *Echinococcus felidis* e *Echinococcus shiquicus* (THOMPSON, 2008; NAKAO et al., 2010, 2013; NAKAO; LAVIKAINEN; HOBERG, 2015; ITO et al., 2016). Entre as espécies reconhecidas, especialmente duas são reconhecidas como de importância médica: *E. multilocularis*, causadora da hidatidose alveolar em humanos e *E. granulosus*, causadora da hidatidose cística.

## 1.2 Hidatidose cística

A hidatidose cística é uma zoonose causada pela infecção com a forma larval (metacestódeo) de *E. granulosus*, sendo reconhecida pela Organização Mundial da Saúde como uma das 17 doenças tropicais negligenciadas (WHO, 2015). É caracterizada pela formação de um ou vários cistos hidáticos uniloculares que se desenvolvem principalmente no fígado e pulmões dos hospedeiros intermediários.

A doença apresenta um longo período entre a infecção e o surgimento de sintomas, o que pode dificultar o diagnóstico e o tratamento (ROGAN et al., 2006; MANDAL; DEB MANDAL, 2012; YANG; ELLIS; MCMANUS, 2012). Inicialmente os cistos são pequenos e os pacientes são assintomáticos, mas à medida que os cistos aumentam de tamanho, eles podem exercer pressão sobre os órgãos vizinhos e causar alterações patológicas, como icterícia, febre e dor abdominal, que são os sintomas mais comuns. Outras complicações que podem ocorrer são o aumento do fígado (hepatomegalia), o aumento da pressão arterial no sistema porta (hipertensão portal) e a inflamação do canal biliar (colangite). Em caso de ruptura do cisto, pode ocorrer reação anafilática em resposta à liberação de líquido hidático ou, ainda, a formação de cistos hidáticos secundários causados pela liberação de protoescólices.

O diagnóstico é baseado em exames de imagem, como tomografia, ultrassonografia e ressonância magnética (MORO; SCHANTZ, 2009; MANDAL; DEB MANDAL, 2012; YANG; ELLIS; MCMANUS, 2012). Após a detecção do cisto, testes serológicos podem ser utilizados para a confirmação do diagnóstico. O tratamento da doença envolve a remoção cirúrgica do cisto ou de seu conteúdo por punção e aspiração, ou o uso de quimioterapia com benzimidazois (MCMANUS et al., 2003; ROGAN et al., 2006).

Os parasitos do gênero apresentam uma ampla distribuição geográfica, com focos endêmicos presentes em todos os continentes habitados (Figura 1). A maior prevalência está localizada em países de zonas temperadas, incluindo partes da Eurásia (regiões mediterrâneas, centro e sul da Rússia, Ásia central, China), Austrália, algumas partes da América (especialmente a América do Sul) e norte e leste da África (DAKKAK, 2010; KNIGHT-JONES; MYLREA; KAHN, 2010; AKRITIDIS, 2011; ROMIG et al., 2011; GROSSO et al., 2012; CUCHER et al., 2015).

No Rio Grande do Sul, segundo o Departamento de Produção Animal (DPA) da Secretaria da Agricultura, Pecuária, Pesca e Agronegócio (SEAPPA-RS), 10% dos bovinos e 25% dos ovinos abatidos em estabelecimentos sob inspeção de fiscais do DPA/SEAPPA-RS, entre 2001 a 2009, apresentavam lesões compatíveis com a presença do parasito. Quanto às

espécies, há relatos da ocorrência de *E. granulosus sensu stricto*, *E. ortleppi* e *E. canadensis* em rebanhos de bovinos e ovinos (BALBINOTTI et al., 2012; URACH MONTEIRO et al., 2016). Especificamente em bovinos, Balbinotti e colaboradores (2012) estimaram que 56,6% das infecções são provocadas por *E. granulosus* e 43,4% por *E. ortleppi*.

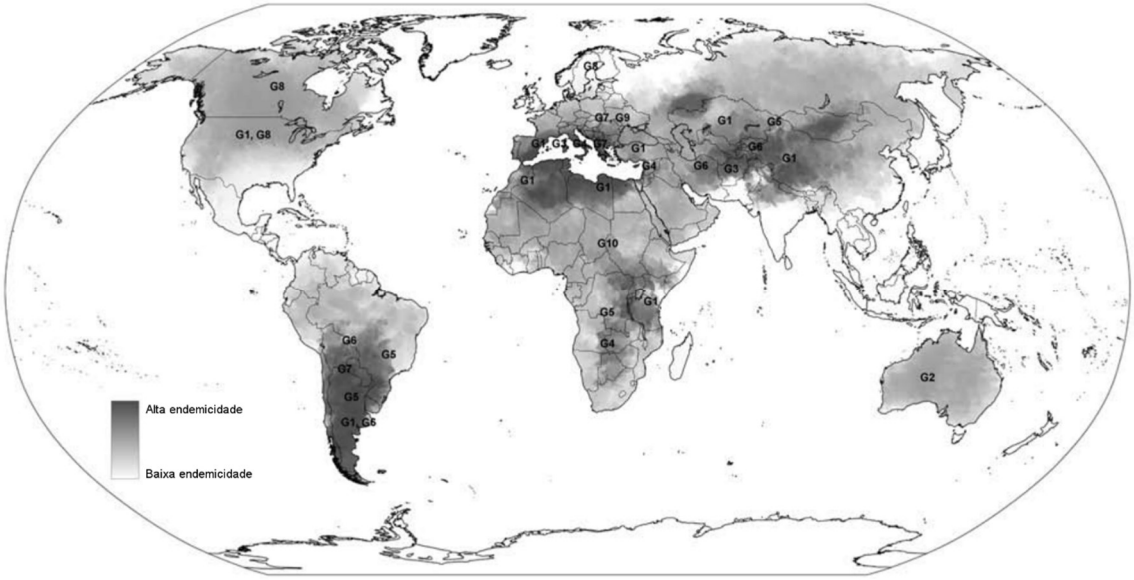


**Figura 1. Endemicidade e distribuição mundial dos antigos genótipos de *E. granulosus*.** Adaptado de GROSSO et al. (2012).

## 1.3 Ciclo de vida do *E. granulosus*

O ciclo de vida do *E. granulosus* é considerado doméstico, uma vez que envolve o cão doméstico como principal hospedeiro definitivo e várias espécies de ungulados domésticos ou, acidentalmente, o homem como hospedeiros intermediários. Na fase sexual, o verme adulto hermafrodita reside no intestino do hospedeiro definitivo (Figura 2), enquanto que a fase assexual ocorre em cistos hidáticos presentes em órgãos do hospedeiro intermediário.

A forma adulta mede entre 3 e 6 mm de comprimento e reside no intestino delgado do hospedeiro definitivo (MCMANUS et al., 2003; ECKERT; DEPLAZES, 2004; MORO; SCHANTZ, 2009). É formada por um escólex anterior, responsável pela fixação do parasito ao hospedeiro; e um estróbilo posterior, dividido em proglótides, as quais variam entre si de acordo com a maturação sexual. Após 4-6 semanas de infecção, a proglótide grávida libera ovos, que são eliminados pelo hospedeiro juntamente com as fezes. Ao serem ingeridos por um hospedeiro intermediário, os ovos entram em contato com enzimas proteolíticas, eclodem e liberam a oncosfera. A oncosfera, então, penetra na parede intestinal e migra, via sistema

circulatório, para órgãos como fígado e pulmões, onde se desenvolve em cisto hidático ou metacestódeo.

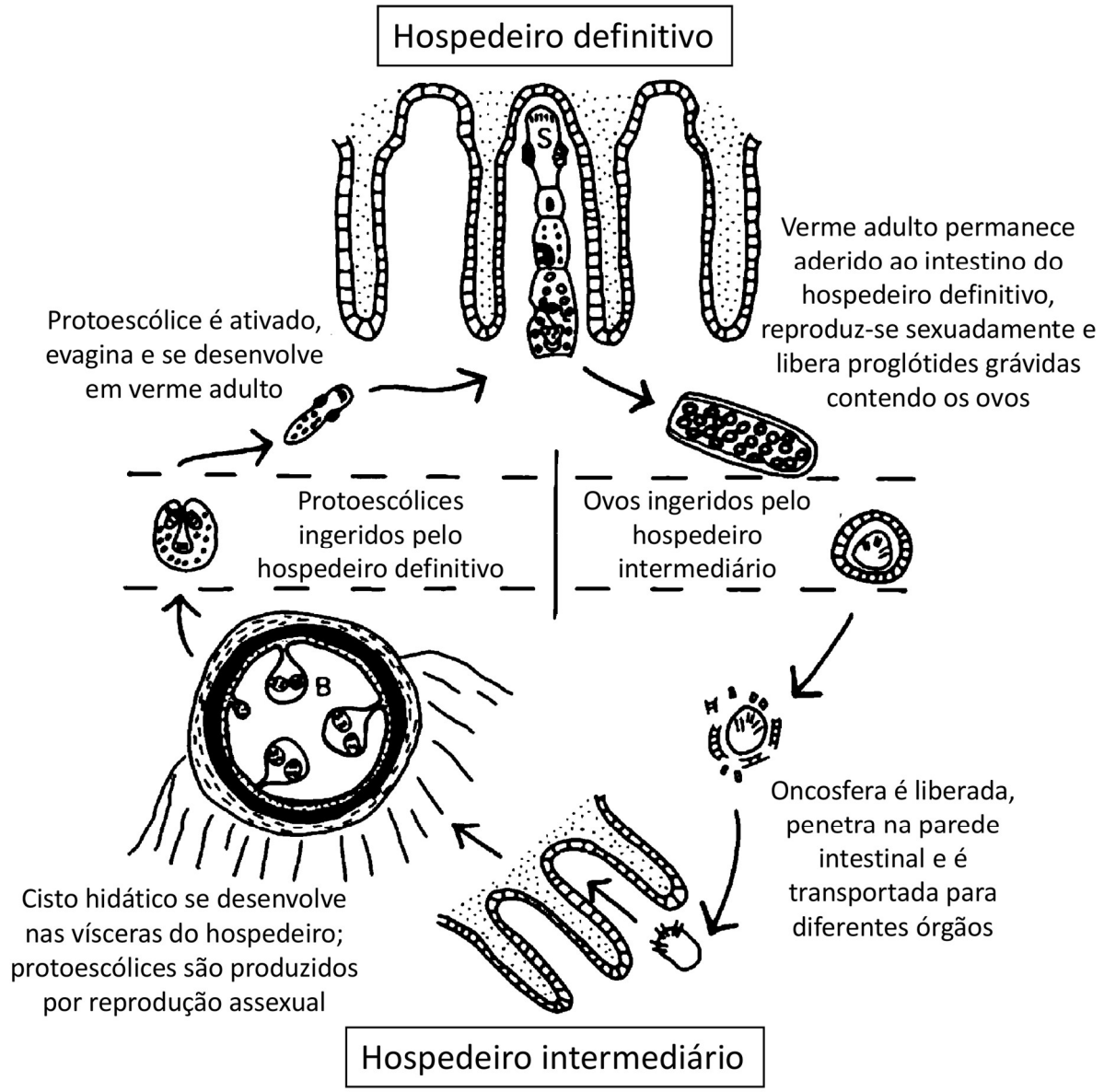


**Figura 2. Ciclo de vida do *E. granulosus*.** Adaptado de THOMPSON (1995).

O cisto hidático possui uma estrutura unilocular preenchida pelo líquido hidático (ROGAN et al., 2006; CASARAVILLA; DÍAZ, 2010; DÍAZ et al., 2011). O cisto é constituído por uma camada laminar acelular externa e uma camada germinativa, mais interna, formada por células indiferenciadas. São essas células as responsáveis pela formação de cápsulas prolígeras contendo os protoescólices. Quando ingerido por um hospedeiro definitivo adequado, os protoescólices evaginam e se desenvolvem em vermes adultos, completando o ciclo de vida do

parasito. Além disso, os protoescólices de *E. granulosus* apresentam uma intrigante capacidade proliferativa, que os permite rediferenciar em cisto hidático secundário. Tais cistos são gerados naturalmente a partir de protoescólices liberados na cavidade intraperitoneal do hospedeiro como resultado da ruptura acidental ou durante a cirurgia para a remoção do cisto primário (ECKERT; DEPLAZES, 2004; MORO; SCHANTZ, 2009).

## 1.4 Desenvolvimento em verme adulto

O desenvolvimento do verme adulto de *Echinococcus* envolve a diferenciação germinativa e somática e pode ser dividido em quatro processos: proglotização, maturação, crescimento e segmentação (THOMPSON et al., 2006; THOMPSON, 2017). A diferenciação germinativa compreende a proglotização, que se refere a formação sequencial de novas unidades reprodutivas (proglótides) e a maturação dessas. Já a diferenciação somática consiste no crescimento e na delimitação de cada proglótide por segmentação. Em estudos sobre o desenvolvimento estrobilar de *E. granulosus* e *E. multilocularis in vitro*, nos quais os vermes cresceram e/ou segmentaram sem apresentar sinais de maturação, ou exibiram proglotização e maturação sem segmentação, foi demonstrado que esses quatro processos de desenvolvimento podem ocorrer de forma independente e que podem estar associados com a transição do metabolismo citosólico para mitocondrial (THOMPSON, 1977, 2017; CONSTANTINE et al., 1998).

De modo geral, antes da ingestão pelo hospedeiro, a região apical do protoescólex (rostelo, ganchos e ventosas) permanece invaginada, o que protege o escólex até que ele receba os estímulos para a estrobilização (SMYTH; MILLER; HOWKINS, 1967; SMYTH, 1969). Embora a natureza precisa desses estímulos não seja completamente conhecida, acredita-se que a mastigação e a ação da pepsina estomacal tenham papel considerável no processo, além de auxiliarem na remoção das cápsulas prolígeras e restos do cisto. Outros fatores importantes são a presença de condições aeróbicas, a temperatura e a presença de sais biliares.

Uma vez ingerido, o tempo requerido para o protoescólex evaginar é variável (Figura 3), mas a maioria dos indivíduos está evaginado após 6 h (THOMPSON, 1977). Uma vez alongados, os protoescólices tornam-se bastante ativos, visto que precisam rapidamente localizar e se aderir às criptas de Lieberkuhn. Essa fixação é feita principalmente pelas ventosas, ao passo que os ganchos penetram superficialmente na mucosa do epitélio (THOMPSON; DUNSMORE; HAYTON, 1979; THOMPSON; ECKERT, 1983). O sistema nervoso bem desenvolvido e as reversas energéticas de glicogênio são considerados fundamentais para

estimular a motilidade e a adesão (SMYTH, 1967; CAMICIA et al., 2013). Quanto à distribuição dos parasitos, ela ocorre de forma desigual ao longo do intestino, sendo que a maioria dos parasitos ficam localizados na região proximal (CONSTANTINE et al., 1998; THOMPSON et al., 2006).

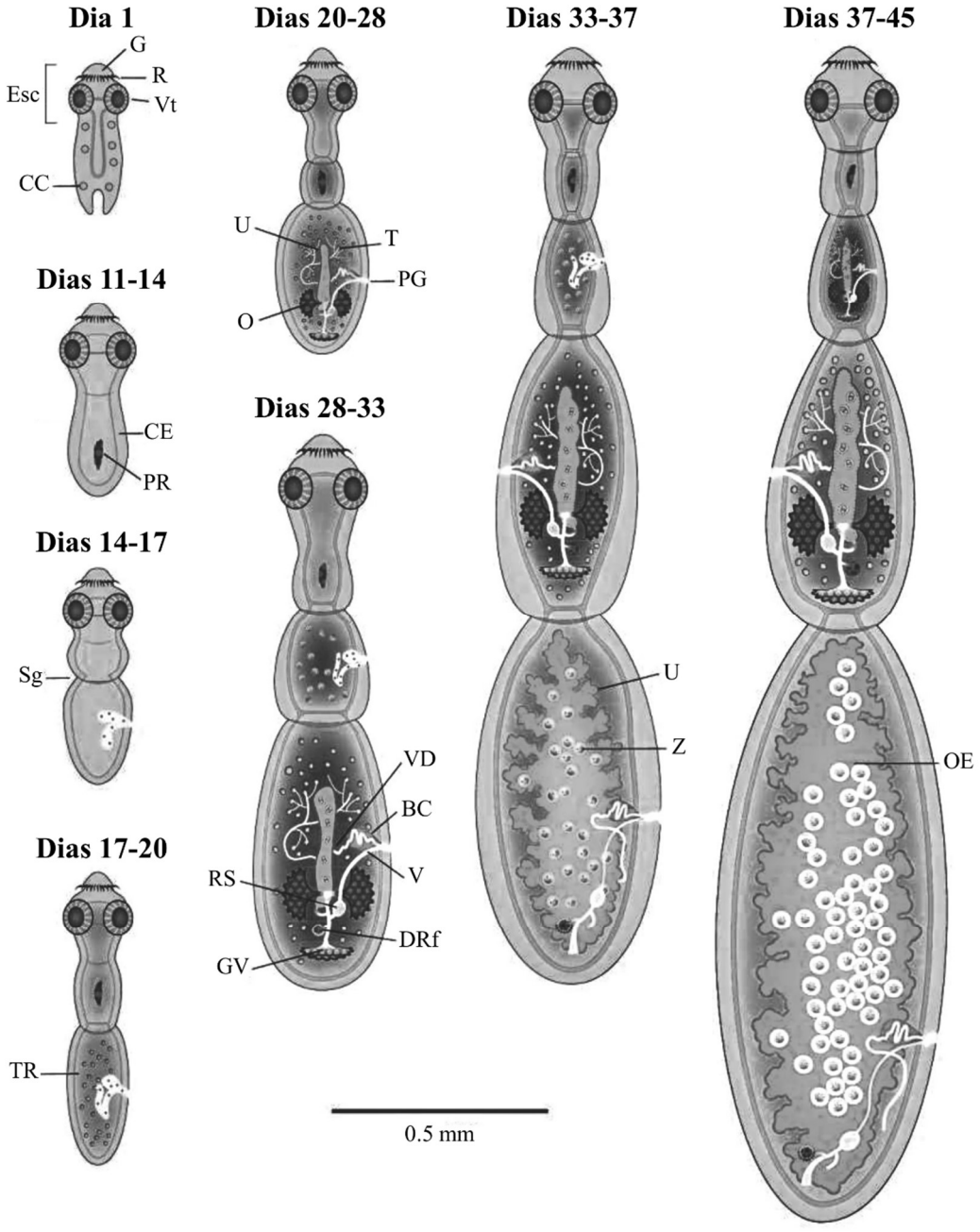


**Figura 3. Estágios de desenvolvimento do verme adulto de *E. granulosus*.** Dia 1: protoescólices evaginados e alongados, contendo numerosos corpúsculos calcários. Dias 11 – 14: diminuição dos corpúsculos calcários, formação dos canais excretores laterais e surgimento do poro genital rudimentar, que indica a formação da primeira proglótide. Dias 14 – 17: primeiro segmento formado. Dias 17 – 20: testículos rudimentares aparecem na primeira proglótide e início da formação da segunda proglótide. Dias 20 – 28: parasito com dois

segmentos. Dias 28 – 33: órgãos masculinos e femininos completamente formados. Dias 33 – 37: ovulação e fertilização na proglótide terminal. Dias 37 – 45: ovos embrionados presentes no útero da última proglótide. *BC*, bolsa de cirro; *CC*, corpúsculo calcário; *CE*, canais excretores; *DRf*, ducto reprodutor feminino; *Esc*, escólex; *G*, gancho; *GV*, glândula vitelina; *O*, ovário; *OE*, ovos embrionados; *PG*, poro genital; *PR*, poro genital rudimentar; *T*, testículo; *TR*, testículo rudimentar, *R*, rostelo; *RS*, receptáculo seminal; *Sg*, segmentação; *U*, útero; *V*, vagina; *VD*, vaso deferente; *Vt*, ventosa; *Z*, zigoto. Adaptado de THOMPSON (2017).

Os protoescólices recém evaginados apresentam um número significativo de corpúsculos calcários, constituídos por uma matriz orgânica e material inorgânico (SMYTH, 1969). Eles seriam uma fonte de íons inorgânicos, $CO_2$ e fosfatos, além de neutralizarem os produtos finais do metabolismo anaeróbico e proteger contra os ácidos gástricos encontrados durante a passagem pelo estômago do hospedeiro definitivo (SMITH; RICHARDS, 1993). Após 3 a 5 dias de infecção, esses corpúsculos desaparecem e os canais excretores tornam-se evidentes (SMYTH; DAVIES, 1974; SMYTH, 1990).

Ao final da primeira semana, uma bexiga excretora posterior também é visualizada. Os primeiros sinais de proglotização são verificados com o surgimento do poro genital rudimentar e o um sítio da constrição, por volta de 11 dias após a infecção. Aos 14 dias, a segmentação torna evidente a primeira proglótide, que, em cestódeos, não envolve a formação de membranas (MEHLHORN et al., 1981). A demarcação entre as sucessivas proglótides é provocada por um dobramento do tegumento, que resulta na aparência constrita.

Entre os dias 17 e 20, surgem sinais dos testículos na primeira proglótide e é iniciada a formação da segunda proglótide. O *E. granulosus* é hermafrodita e capaz de realizar tanto a fertilização cruzada quanto a autofertilização, sendo esta a mais frequente (SMYTH; SMYTH, 1969). O hermafroditismo e a autofertilização são consideradas vantagens para o parasito, que poderia ter dificuldade em encontrar outro parasito, principalmente em infecções mais brandas. A produção de ovos ocorre a partir de 34-58 dias após a infecção (SMYTH; DAVIES, 1974; THOMPSON et al., 2006).

## 1.5 Expressão gênica em *E. granulosus*

Dois genomas da espécie *E. granulosus* sensu stricto foram sequenciados e disponibilizados em bancos de dados públicos (Tabela 1). O primeiro (PRJEB121), contém as sequências de dois cistos hidáticos de genótipo G1 obtidos de pulmão de bovino naturalmente

infectado no Uruguai, e que foi conduzido pelo *Wellcome Trust Sanger Institute* (TSAI et al., 2013); o segundo (PRJNA182977), refere-se a um único cisto hidático de genótipo G1 obtido de um fígado de ovelha da China e sequenciado pelo *Chinese National Human Genome Center* (ZHENG et al., 2013). Tsai et al (2013) também publicaram o genoma de *Taenia solium*, *Hymenolepis microstoma* e *E. multilocularis*, sendo esta última, a espécie escolhida para a produção de um genoma de referência de alta qualidade e que serviu como base para a montagem do genoma de *E. granulosus*. A busca e anotação de genes foi amparada por análises do transcritoma de diferentes estágios do ciclo de vida, como oncosfera, metacestódeo, protoescólex e verme adulto (FERNÁNDEZ et al., 2002; PARKINSON et al., 2012; TSAI et al., 2013; ZHENG et al., 2013).

**Tabela 1. Propriedades dos genomas de *Echinococcus***

| | *E. multilocularis* | *E. granulosus* | |
|---|---|---|---|
| | PRJEB122[a,b] | PRJEB121[a] | PRJNA182977[a] |
| **Tamanho do genoma (MB)** | 114,9 | 114,5 | 110,8 |
| **Número de genes** | 10663 | 10245 | 11319 |
| **N50 (MB)** | 13,8 | 5,2 | 0,7 |
| $\mathbf{n_{N50}}$ | 4 | 6 | 39 |
| **Maior *scaffold* (MB)** | 20,1 | 16 | 3,9 |
| **N90 (kB)** | 2900 | 213,5 | 127,3 |
| $\mathbf{n_{N90}}$ | 10 | 41 | 181 |

[a] Número de acesso do WormBase ParaSite (http://parasite.wormbase.org/index.html).

[b] Genoma de referência.

Os genomas de *Echinococcus* são cerca de três vezes menores que os de trematódeos e nove vezes menores que os de platelmintos de vida livre. Acredita-se que isso ocorra devido às regiões intergênicas menores, o tamanho menor dos íntrons e ao menor conteúdo de repetições e elementos genéticos móveis (TSAI et al., 2013).

Através da análise comparativa dos genomas de cestódeos, é possível identificar consideráveis perdas e ganhos de genes associados com a adaptação ao parasitismo. Estão ausentes genes cruciais ou mesmo vias inteiras da síntese *de novo* de ácidos graxos, colesterol, pirimidinas, purinas e da maioria dos aminoácidos (TSAI et al., 2013; ZHENG et al., 2013). Por conseguinte, essas moléculas precisam ser captadas do hospedeiro, o que justifica o fato de

que famílias gênicas associadas a essa captação encontram-se expandidas e/ou altamente expressas. Entre os genes mais expressos no metacestódeo de *Echinococcus*, por exemplo, estão os que codificam para a proteína de ligação a ácidos graxos (FABP) e a família de genes do antígeno B, envolvidos na aquisição e transporte de lipídeos (TSAI et al., 2013; KOZIOL; BREHM, 2015). Entre as expansões, uma das mais significativas em *Echinococcus* é a da família de proteínas de choque térmico HSP70, a qual apresenta 22 cópias, contrastando com as 2 cópias presentes no genoma humano e 6 cópias no genoma de *Drosophila melanogaster* (TSAI et al., 2013; ZHENG et al., 2013).

Por outro lado, alguns sistemas de sinalização apresentam-se evolutivamente conservados, como é o caso dos componentes do fator de crescimento epidérmico (EGF), fator de crescimento fibroblástico (FGF), fator de crescimento transformador β (TGF-β) e a cascata de transdução de sinal da insulina (BREHM, 2010; TSAI et al., 2013; ZHENG et al., 2013).

Em relação aos genes de vias de desenvolvimento relacionados com a complexidade do plano corporal, houve uma redução significativa em cestódeos, os quais apresentam o menor número de genes homeobox entre os animais bilatérios. Especificamente, foram perdidos vários genes ParaHox, envolvidos na especificação de um intestino (KOZIOL; LALANNE; CASTILLO, 2009; TSAI et al., 2013). Além disso, vários genes da família homeobox envolvidos no desenvolvimento neural também estão ausentes (TSAI et al., 2013), apesar do *E. granulosus* ter um sistema nervoso bem desenvolvido (BROWNLEE et al., 1994; FAIRWEATHER et al., 1994; CAMICIA et al., 2013; KOZIOL; KROHNE; BREHM, 2013). Por fim, ainda que os genomas de *Echinococcus* aparentemente codifiquem conjuntos típicos de genes Hedgehog e Notch, eles exibem um conjunto reduzido de genes relacionados à via de sinalização Wnt (RIDDIFORD; OLSON, 2011; TSAI et al., 2013).

Uma característica interessante do *Echinococcus* que o difere de seu hospedeiro é o mecanismo de *trans-splicing* (BREHM; JENSEN; FROSCH, 2000). O *trans-splicing* é um mecanismo de processamento do RNA mensageiro (mRNA) que envolve a fusão de éxons de transcritos primários independentes para formar o mRNA maduro. No tipo mais comum de trans-splicing, chamado de *spliced leader* (SL), um pequeno éxon é adicionado à extremidade 5’ das moléculas de pré-mRNA. Dessa forma, todos os transcritos que sofrem esse processo apresentam um éxon idêntico na sua extremidade 5'. A função biológica do *trans-splicing* permanece desconhecida, embora uma das hipóteses sugira que se trata de um processo adaptativo para a coordenação da regulação gênica ou controle traducional. Análises genômicas e transcritômicas revelaram que aproximadamente 13% dos os genes de *E. multilocularis* sofrem o processo de *trans-splicing*, dos quais centenas estão dispostos em policistrons de até

quatro genes (TSAI et al., 2013). Entre eles, estão genes envolvidos em processos celulares essenciais como o controle transcricional e traducional, o *splicing* e a replicação (BREHM; JENSEN; FROSCH, 2000; TSAI et al., 2013). Em *E. granulosus*, verificou-se a presença de *trans-splicing* em genes relacionados ao metabolismo energético e a antioxidantes (PARKINSON et al., 2012).

## 1.6 *E. granulosus* como um modelo de estudo

Grande parte do conhecimento adquirido acerca da biologia do *E. granulosus* foi obtido a partir de estudos com o metacestódeo, o ponto de partida lógico para os experimentos, uma vez que é encontrado naturalmente em animais infectados abatidos em frigoríficos. Entre as dificuldades encontradas para a utilização de outros estágios do parasito e que limitaram as investigações *in vivo* estão as dificuldades práticas e éticas envolvidas na manutenção de infecções em hospedeiros definitivos (cães) e a ausência de um hospedeiro alternativo que possa ser mantido em laboratório para a manutenção de infecções com o parasito adulto (THOMPSON et al., 2006).

Essas dificuldades estimularam a busca pelo estabelecimento de condições de cultivo *in vitro*. Entretanto, independentemente do sistema de cultivo utilizado, os primeiros trabalhos mostraram que os protoescólices sempre diferenciavam na direção cística (YAMASHITA et al., 1962; WEBSTER; CAMERON, 1963; SMYTH, 1967). Somente a partir de estudos histológicos durante as fases iniciais da infecção no hospedeiro definitivo é que foi postulada a hipótese de que, antes do início do desenvolvimento estrobilar, a região anterior de um protoescólex deveria se ligar ou, pelo menos, fazer contato com um substrato da mesma natureza daquele presente na superfície do intestino dos cães (SMYTH; HOWKINS; BARTON, 1966; MORSETH, 1967; SMYTH; GEMMELL; SMYTH, 1970). Dessa forma, os pesquisadores passaram a utilizar um meio bifásico, com base proteica coagulada para desencadear as condições fisiológicas necessárias ao desenvolvimento estrobilar. Com o avanço nas pesquisas, outras substâncias foram incorporadas ao meio de cultivo. O uso da pepsina e de sais biliares, por exemplo, permitiram um significativo aumento na porcentagem de evaginação (MACPHERSON; SMYTH, 1985; SMYTH; DAVIES, 1974; e resumidas em SMYTH, 1990).

Como mostrado anteriormente na figura 3, esses estudos propiciaram o estabelecimento de padrões de desenvolvimento, ou seja, critérios morfológicos que possibilitam avaliar as condições de cultivo utilizadas. Tais padrões abrangem desde a evaginação e diminuição dos

corpúsculos calcários observados nos primeiros dias de cultivo, até a segmentação e surgimento de características sexuais.

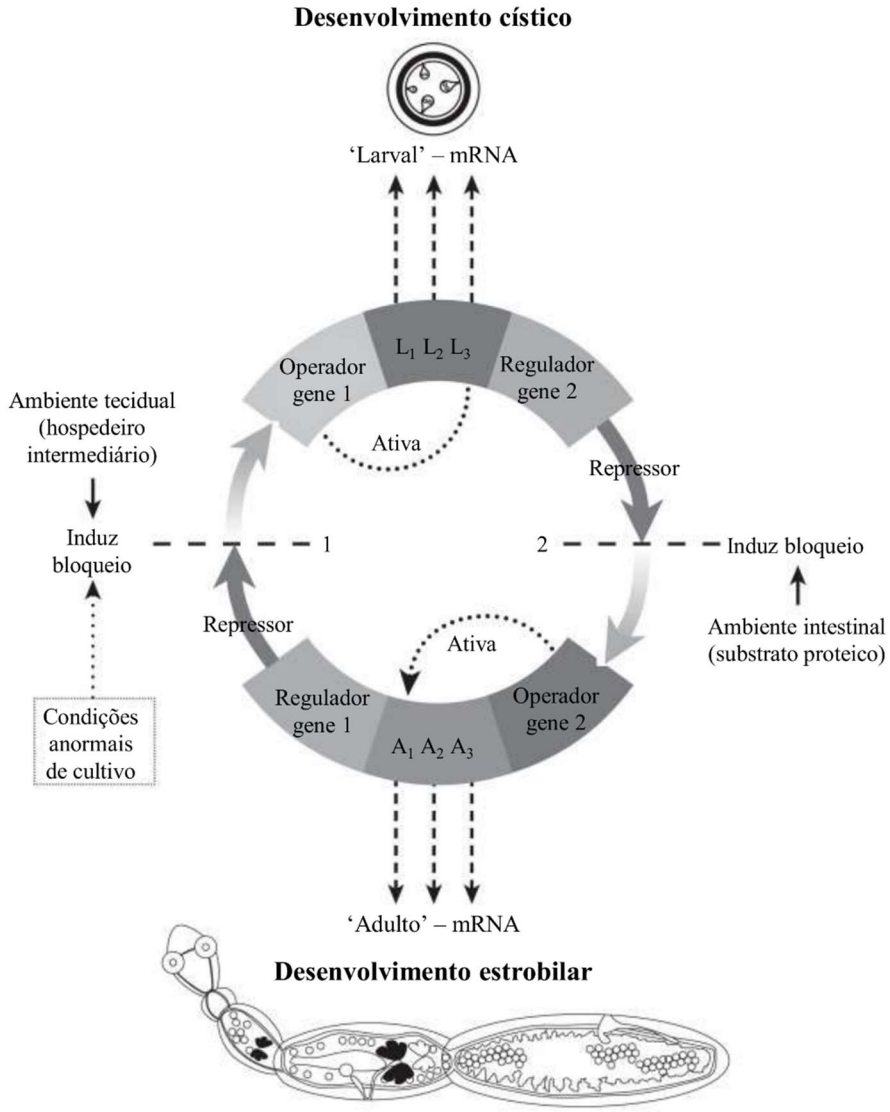


**Figura 4. Modelo de controle da diferenciação em *Echinococcus*.** Modelo hipotético simplificado de regulação da expressão gênica, baseado no modelo Jacob-Monod, para diferenciação de *Echinococcus granulosus* em forma cística (larval) ou estrobilar (adulto). L1-3, A1-3, genes estruturais larvais e adultos. Adaptado de THOMPSON & LYMBERY (2013).

James D. Smyth foi um dos primeiros pesquisadores a demonstrar o potencial de *E. granulosus* como organismo modelo para estudos relacionados à biologia do desenvolvimento, diferenciação e interações parasito-hospedeiro (SMYTH; HOWKINS; BARTON, 1966; SMYTH; MILLER, 1967; SMYTH; MILLER; HOWKINS, 1967). É dele um dos primeiros relatos sobre a intrigante capacidade dos protoescólices se desenvolverem em duas direções

distintas (cisto ou verme adulto), ainda na década de 1960 (SMYTH; HOWKINS; BARTON, 1966). Foi ele, também, quem desenvolveu um modelo hipotético (Figura 4), porém lógico, para explicar como os genes regulam o desenvolvimento em *Echinococcus* (SMYTH, 1969). Baseado no modelo de expressão gênica de Jacob-Monod, são apresentadas as complexas interações que podem estar envolvidas no controle do desenvolvimento e diferenciação em *Echinococcus*. Smyth foi, ainda, pioneiro na exploração de *Echinococcus* como um novo modelo de estudo do parasitismo de forma distinta dos estudos de avaliação de drogas anti-helmínticas ou antiparasitárias, para englobar estudos de natureza mais fundamental na biologia.

## 2. Justificativa e Objetivos

Importantes mudanças morfológicas e bioquímicas ocorrem durante o ciclo de vida dos parasitos e são, muito provavelmente, o resultado de alterações reguladas na expressão gênica em resposta a estímulos ambientais, como a mudança de hospedeiro, temperatura e pH. No caso do *E. granulosus*, por ter um ciclo de vida complexo, que necessita de dois hospedeiros distintos, a regulação dos genes que serão expressos e/ou silenciados em cada etapa possivelmente contribui para as alterações fenotípicas do parasito associadas aos diferentes estágios do ciclo de vida.

Os recentes trabalhos de genoma (TSAI et al., 2013; ZHENG et al., 2013), transcritoma (PARKINSON et al., 2012; PAN et al., 2014) e proteoma (CUI et al., 2013) acrescentam informações relevantes na comparação entre os diferentes estágios do parasito, mas pouco ou nada contribuem para a elucidação dos eventos moleculares envolvidos no período de transição entre eles. Nesse aspecto, estudos direcionados de proteômica e transcritômica fornecem uma quantidade significativa de informação, a qual pode ser útil não só para uma compreensão mais profunda da biologia molecular e fisiologia do parasito, bem como da patogênese na hidatidose cística.

Dessa forma, o objetivo deste trabalho é contribuir para o melhor entendimento dos mecanismos moleculares modulados durante a indução *in vitro* ao desenvolvimento estrobilar de protoescólices de *E. granulosus* através da marcação e detecção de proteínas recém-sintetizadas e da análise do transcritoma.

Especificamente, objetivamos:

- A padronização das condições de cultivo *in vitro* para a indução ao desenvolvimento estrobilar de protoescólices de *E. granulosus*;

- A marcação e identificação de proteínas recém-sintetizadas através da incorporação de azidohomoalanina e análise por espectrometria de massas;

- A identificação de genes expressos durante as primeiras horas de indução à estrobilização através de RNA-seq e análises de bioinformática.

## **3. Capítulo I** - Identification of newly synthesized proteins by *Echinococcus granulosus* protoscoleces upon induction of strobilation

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Contribuição dos autores:

JAD: delineamento experimental, execução dos experimentos (exceto operação do espectrômetro de massas), análise e discussão dos resultados e redação do manuscrito; HBF, HM: espectrometria de massas e revisão do manuscrito; JRB: revisão do manuscrito; KMM, AZ: delineamento experimental, análise e discussão dos resultados e redação do manuscrito.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

# Identification of Newly Synthesized Proteins by *Echinococcus granulosus* Protoscoleces upon Induction of Strobilation

**João Antonio Debarba[1,2], Karina Mariante Monteiro[1,2,3], Hercules Moura[4], John R. Barr[4], Henrique Bunselmeyer Ferreira[1,2,3], Arnaldo Zaha[1,2,3]***

**1** Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil, **2** Laboratório de Biologia Molecular de Cestódeos, and Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil, **3** Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, **4** Biological Mass Spectrometry Laboratory, Clinical Chemistry Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America

* zaha@cbiot.ufrgs.br



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## Abstract

### Background

The proteins responsible for the key molecular events leading to the structural changes between the developmental stages of *Echinococcus granulosus* remain unknown. In this work, azidohomoalanine (AHA)-specific labeling was used to identify proteins expressed by *E. granulosus* protoscoleces (PSCs) upon the induction of strobilar development.

### Methodology/Principal Findings

The *in vitro* incorporation of AHA with different tags into newly synthesized proteins (NSPs) by PSCs was analyzed using SDS-PAGE and confocal microscopy. The LC-MS/MS analysis of AHA-labeled NSPs by PSCs undergoing strobilation allowed for the identification of 365 proteins, of which 75 were differentially expressed in comparison between the presence or absence of strobilation stimuli and 51 were expressed exclusively in either condition. These proteins were mainly involved in metabolic, regulatory and signaling processes.

### Conclusions/Significance

After the controlled-labeling of proteins during the induction of strobilar development, we identified modifications in protein expression. The changes in the metabolism and the activation of control and signaling pathways may be important for the correct parasite development and be target for further studies.

## Author Summary

In the life cycle of the parasite *Echinococcus granulosus*, hydatid cysts produce the pre-adult form, which has the ability to either differentiate into an adult worm (strobilation) or dedifferentiate into a secondary hydatid cyst. We used different protein tags that allowed for the visualization and purification of proteins produced specifically after the induction of strobilar development to identify proteins that might be involved in this process (temporally controlled and context-dependent). As a result, we found proteins that are involved in important processes during development, such as energy metabolism, control pathways and cellular communication. We believe that these results will be useful for the development of scientific approaches to controlling and preventing cystic hydatid disease.

## Introduction

The parasite *Echinococcus granulosus* is a cestode tapeworm that acts as the causative agent of cystic echinococcosis (cystic hydatid disease), one of the 17 neglected tropical diseases to be recently prioritized by the World Health Organization [1]. During its life cycle, the *E. granulosus* adult worm resides in the intestine of the definitive host (e.g., dogs), releasing their eggs with the host feces. Following ingestion by the intermediate host (e.g., domestic ungulates), the eggs release oncospheres that penetrate the intestinal wall and then migrate to various organs of the host. At the organ site, the oncosphere develops in the larval stage of the parasite, the hydatid cyst (metacestode). The pre-adult forms (protoscolex, PSC) are asexually formed in the cyst germinal cellular layer and liberated into the lumen of hydatid cysts [2–7]. In the cyst cavity, PSCs may remain in an inactive state for years until the structural integrity of the cyst is lost and they exhibit a dual developmental capacity. When ingested by a definitive host, PSCs sexually differentiate into fully developed, segmented adult worms in a process called strobilation. Alternately, upon hydatid cyst rupture and the release of its contents into the peritoneal cavity of an intermediate host, PSCs can dedifferentiate into secondary hydatid cysts [8].

This dual developmental capacity of the parasite and its requirement for more than one host to complete its life cycle are associated with its ability to readily respond to host environmental changes and regulate its gene expression and protein synthesis [9–11]. Transcriptional and proteomic studies have identified differentially expressed genes and proteins between the different life stages and cyst components of *E. granulosus* [6, 12–14]. However, the identities of the proteins responsible for key molecular events that lead to structural changes of the parasite and its transition between different developmental stages remain essentially unknown. One possible reason for this is the difficulty of indirectly associating changes in gene expression to the response from a particular stimulus. Consequently, the direct visualization and identification of newly synthesized proteins (NSPs) is useful for revealing the spatiotemporal characteristics of proteomes during development [15].

Recently, the application of bioorthogonal non-canonical amino acid tagging (BONCAT) and fluorescent non-canonical amino acid tagging (FUNCAT) have been described for the non-radioactive labeling, visualization, purification and identification of NSPs [11, 16, 17]. In BONCAT, newly synthesized proteins containing non-canonical amino acids containing either azide or alkyne moieties, such as the methionine (Met) analogue azidohomoalanine (AHA), are chemically combined with affinity tags. The alkyne or azide functional groups used in BONCAT require further purification steps, whereas FUNCAT uses fluorescent tags for *in situ* visualization. BONCAT has been used for labeling NSPs in response to different stimulus in

mammalian [16, 17] and bacterial [18] cells. Moreover, BONCAT has been used in combination with FUNCAT to show NSPs in zebrafish [19]. Further adaptations have allowed the application of these methods to identifying NSPs in model organisms such as *Caenorhabditis elegans*, fruit fly and mouse [20].

Here, we report the application of FUNCAT and BONCAT followed by confocal, SDS-PAGE and MS analyses to study *E. granulosus* NSPs and to identify 365 AHA-labeled NSPs during the *E. granulosus* strobilar development *in vitro*. Some of the identified proteins have important functions in key processes for the survival and development of the parasite, such as metabolic reactions and processes involved in host/parasite relationships. The further applicability of these methods for developmental studies in parasitic flatworms is also discussed.

## Methods

### PSC collection

Hydatid cysts from *E. granulosus* (G1 genotype) were obtained from the naturally infected livers and lungs of cattle routinely slaughtered in a local abattoir (São Leopoldo, RS, Brazil). Finding hydatid cysts during mandatory animal inspection renders contaminated viscera unfit for human consumption, and *E. granulosus* contaminated livers and lungs were donated by the abattoir for use in this work. PSCs were collected by aspiration, decanted by gravity and washed several times with PBS, pH 7.4 [13]. The viability of PSCs was determined using the trypan blue exclusion test [21] and the motility was evaluated visually using an inverted light microscope. Only PSCs with viability greater than 90% were used for further analysis. The PSCs were genotyped by one-step PCR [22].

### Cultivation of PSCs and the metabolic incorporation of AHA

PSCs were cultured *in vitro* as previously described [23], with minor modifications. Briefly, PSCs were incubated at 37°C and 5% $CO_2$ for 15 min with pepsin (2 mg/mL, Sigma, St. Louis, MO, USA) in Hanks' Balanced Salt Solution (HBSS), pH 2.0. The PSCs were then washed three times with PBS containing antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin, Sigma, St. Louis, MO, USA) before being incubated with the appropriate medium. To stimulate the PSCs to undergo strobilar development (SSD), the PSC suspension was transferred to a biphasic medium. Approximately 500 PSCs were used per mL of liquid medium over the solid base. The biphasic medium contained coagulated newborn calf serum (Gibco, Auckland, NZ) as the solid phase, which was obtained by heating the serum at 76°C in a water bath for 10 to 30 min. Each 100 mL of the liquid phase consisted of 83.5 mL RPMI without Met (Gibco, Grand Island, NY, USA), 15 mL fetal bovine serum (Vitrocell, Campinas, SP, BR), 1.15 mL glucose (Merck, Loughborough, UK) 30% in 18 megaOhm water, 0.35 mL taurocholate 0.2% (Sigma, St. Louis, MO, USA) in HBSS, 100 IU/mL penicillin/streptomycin and AHA (Invitrogen, Eugene, OR, USA) in a final concentration of 50 μM. The control without stimuli for strobilar development (NSD) consisted of PSCs maintained in a monophasic medium containing RPMI without Met, 15% of fetal bovine serum and AHA (50 μM). The negative control (NC) for AHA incorporation consisted of the NSD condition without AHA. The cultures were maintained at 37°C and 5% CO2 for 24 h for the proteome analysis or 72 h for the other experiments.

### Detection of newly synthesized proteins

**SDS-PAGE.** Cell lysis and protein extraction were performed using the Click-It Metabolic Labeling Reagents for Proteins kit (Invitrogen, Eugene, OR, USA) according to manufacturer's

instructions, with modifications. Briefly, the PSCs were transferred from the culture medium to a 1.5 mL tube and washed three times with PBS. Then, 200 μL of lysis buffer (1% SDS in 50 mM Tris-HCl, pH 8.0) were added per 1500 PSCs. After 15 min of incubation on ice, the PSCs were gently homogenized using a needle and a syringe. The lysate was sonicated for 3 x 30 sec with a 60 sec interval between pulses, vortexed for 5 min and centrifuged at 13,000×*g* at 4°C for 5 min. The protein concentration was determined by fluorometry using the Qubit Protein Assay Kit (Invitrogen, Carlsbad, CA, USA), followed by precipitation with methanol/chloroform.

The proteins were solubilized in lysis buffer and 200 μg were labeled using the Click-iT Tetramethylrhodamine (TAMRA) alkyne Protein Analysis Detection Kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions. After labeling, the proteins were precipitated with methanol/chloroform, resuspended in Thiourea/Urea buffer (7 M urea, 2 M thiourea, 4% CHAPS) and separated in 12% SDS-PAGE. The TAMRA-labeled proteins were visualized under 300 nm ultraviolet (UV) light.

**Confocal microscopy.** To visualize the spatial distribution of AHA-labeled NSPs, whole mount PSCs were fixed at room temperature with 4% paraformaldehyde (in PBS) for 30 min. The PSCs were permeabilized with proteinase K (20 μg/mL) for 20 min, followed by two 5-min washes in PBS/0.1 M glycine, two 15-min washes in PBS/0.1% Triton X-100 and a final 10-min wash in PBS/1% BSA before conjugation with Alexa Fluor 488 Alkyne using the Click-iT Cell Reaction Buffer Kit (Invitrogen, Eugene, OR, USA). Subsequently, the DNA was stained with 50 mM 4',6-diamidino-2-phenylindole (DAPI) in PBS-Tween 0.05% containing 1% BSA for 20 min. Fluoromount (Sigma, St. Louis, MO, USA) and coverslips were used to preserve the PSCs. Images were obtained using an Olympus FluoView 1000 confocal microscope and the post-acquisition processing and analysis were performed using ImageJ (NCBI, NIH). One-Way Anova statistical analysis was performed, assuming $p<0.05$ as significant.

## Identification of newly synthesized proteins

**Sample preparation and mass spectrometry.** The PSC protein extraction was performed according to the Click-iT Protein Enrichment Kit (Invitrogen, Eugene, OR, USA) manufacturer's instructions. Briefly, the PSCs were incubated in urea lysis buffer (8 M urea, 200 mM Tris pH 8.0, 4% CHAPS, 1 M NaCl) supplemented with protease inhibitors (Sigma, St. Louis, MO, USA) on ice for 10 min. Then, the lysate was sonicated for 3 x 30 sec with a 60 sec interval between pulses, vortexed for 5 min and centrifuged at 10,000×*g* at 4°C for 5 min. An 800 μL aliquot of supernatant was used for click chemistry reactions with alkyne agarose resin. Protein reduction with DTT at 70°C and alkylation with iodoacetamide at room temperature were performed before the resin was washed with 5 x 2 mL SDS Wash Buffer, 10 x 2 mL 8 M urea and 10 x 2 mL 20% acetonitrile for the stringent removal of non-specifically bound proteins. The resin-bound proteins were transferred to a clean tube and digested with trypsin (10 μL of 0.1 μg/μL, Promega, Madison, WI, USA) in approximately 200 μL of digestion buffer (100 mM Tris, 2 mM $CaCl_2$, 10% acetonitrile). After a 5-min centrifugation at 1,000×*g*, the supernatant was treated with 2% acetonitrile, acidified with trifluoroacetic acid and desalted in HLB cartridges (Waters, Milford, MA, USA). The peptides were then eluted with 50% acetonitrile/0.1% TFA, quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) at 205 nm and lyophilized in a SpeedVac concentrator.

The peptides were reconstituted using 0.1% formic acid (Thermo Scientific, Rockford, IL) in water (Burdick and Jackson, Muskegon, MI), loaded onto a nanoAcquity UPLC system (Waters Corporation, Milford, MA) and separated using a gradient elution. The mobile phase solvents consisted of (solvent A) 0.1% formic acid in water and (solvent B) 0.1% formic acid in

acetonitrile (Burdick and Jackson). The gradient flow was set at 300 nl/min. The profile 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% for 5 min before returning to 5% B in 5 min and re-equilibration at 5% B for 20 min. After chromatography, the peptides were introduced into 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 mass spectrometer was programmed to select the top 15 most intense ions with two or more charges. The experiment was performed in two biological and two technical replicates. Each biological sample was composed of PSCs collected from one hydatid cyst.

**Data analysis.** The MS/MS raw data were processed using msConvert (ProteoWizard, version 3) [24], and the peak lists were exported in the Mascot Generic Format (.mgf). The MS/MS data were analyzed using Mascot Search Engine (Matrix Science, version 2.3.02) against a local *E. granulosus* database (21764 sequences) containing the deduced amino acid sequences from the genome annotation available on GeneDB [25] and the Chinese National Human Genome Center at Shanghai (CHGCS) [6]. The search parameters included a fragment ion mass tolerance of 1 Da and a peptide ion tolerance of 10 ppm. Carbamidomethylation was specified as a fixed modification, whereas the oxidation of methionine was specified as a variable modification.

Scaffold (Proteome Software Inc., version 4.4.1) was used to validate the peptide and protein identifications. The peptide identifications were accepted if they could be established at greater than 95.0% probability as assigned by the Peptide Prophet algorithm [26]. The protein identifications were accepted if they could be established at greater than 99% probability as assigned by the Protein Prophet algorithm [27] and contained at least 2 identified peptides. The false discovery rate, FDR (Decoy), was 0.0% for proteins and peptides. The normalized spectral abundance factor (NSAF) [28] was calculated for each protein, and the quantitative differences were statistically analyzed using Student's t-test in Scaffold. The differences with p-values lower than 0.05 were considered statistically significant. Proteins with p-value $< 0.05$ were selected for the hierarchical clustering analysis. A heat map graphical representation was performed in Perseus software (MaxQuant, version 1.5.1.6.) [29]. Protein NSAFs were normalized by their Z-scores and clustered using the Euclidean distance method with average linkages.

The eggNOG database (version 4.1, http://eggnogdb.embl.de/#/app/home) [30] was used to acquire the functional annotation for the identified proteins.

## Results

We have established an *in vitro* model of *E. granulosus* PSC strobilar development under low potential sources of Met and conditions that promote the efficient incorporation of AHA. This treatment has kept the characteristic morphological changes of strobilar development without affecting viability (S1 and S2 Figs; S1 Text). In addition, we analyzed the genomic data for *E. granulosus* to confirm the feasibility of AHA labeling because proteins with non-terminal Met residues are required for this purpose (S1 Text).

### Detection of newly synthesized proteins detection

The proteins synthesized by *E. granulosus* PSCs after 72 h of cultivation in the presence of AHA (SSD) were labeled with TAMRA and visualized under UV to verify their electrophoretic profiles. The pattern of bands in the total extract of PSCs was assessed by inspection of the gels stained with coomassie blue (Fig 1A). The NSPs of the PSCs could be visualized by UV in the

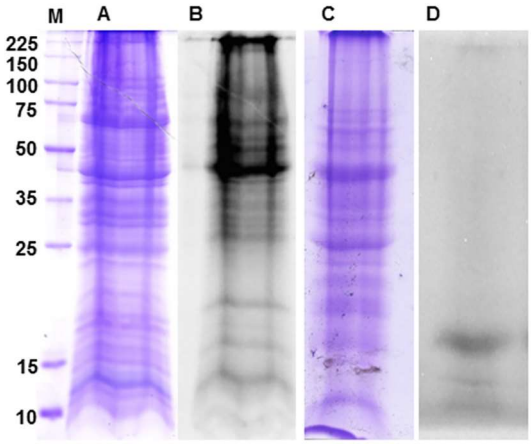


**Fig 1. Detection of NSPs from *in vitro* cultured *E. granulosus* PSCs.** The coomassie-stained proteins and UV detected TAMRA-labeled NSPs from the PSCs incubated for 72 h in the presence (A and B) or absence (C and D) of AHA, respectively.

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same gel. The analysis of the NSPs revealed a complex but lower number of distinct banding patterns that ranged from 10 to 225 kDa (Fig 1B). In the negative control condition without AHA, the total proteins were visualized with coomassie blue (Fig 1C), but no band corresponding to NSPs was visualized under UV (Fig 1D) except for a non-incorporated TAMRA residual band, indicating the high specificity of the reaction.

To evaluate whether the AHA-labeling methodology could be applied to identifying NSPs from *in toto* PSCs and to verify whether the labeled proteins were associated with a particular site or structure in PSCs undergoing strobilation (SSD), Alexa Fluor 488-labeled AHA-containing NSPs were analyzed in whole mount PSCs by confocal microscopy after 72 h of *in vitro* culture (Fig 2). The fluorescence signal corresponding to the labeled NSPs was widely distributed and could be detected all over the PSCs (Fig 2A), though there was a possible correlation between the NSPs and the suckers. In contrast, no NSP-associated fluorescence could be detected in the control experiments. The levels of DAPI fluorescence remained stable under the different conditions (Fig 2B–2D). The estimated fluorescence for the NSP-Alexa Fluor and DAPI are shown in Fig 1E.

## Identification of newly synthesized proteins

We next explored the identities of the NSPs expressed in PSCs undergoing strobilation over a 24-h time window. As we searched for proteins involved in the strobilation process, we chose a shorter culture time because longer times may dilute the importance of these molecules. Scaffold was used to validate 365 non-redundant proteins in the AHA samples (Fig 3). In the non-AHA samples, we found 14 proteins that were excluded from subsequent analyzes. The purified NSPs from PSCs either with strobilation stimuli (SSD) or without strobilation stimuli (NSD) were identified by LC-MS/MS, which allowed for the identification of 248 and 275 proteins, respectively. A list of the newly synthesized proteins identified in both samples is provided in S1 Table.

We found 51 proteins (23 SSD and 28 NSD) in only one of the conditions studied in at least two replicates. We normalized the other 233 identified proteins by NSAF and applied Student's

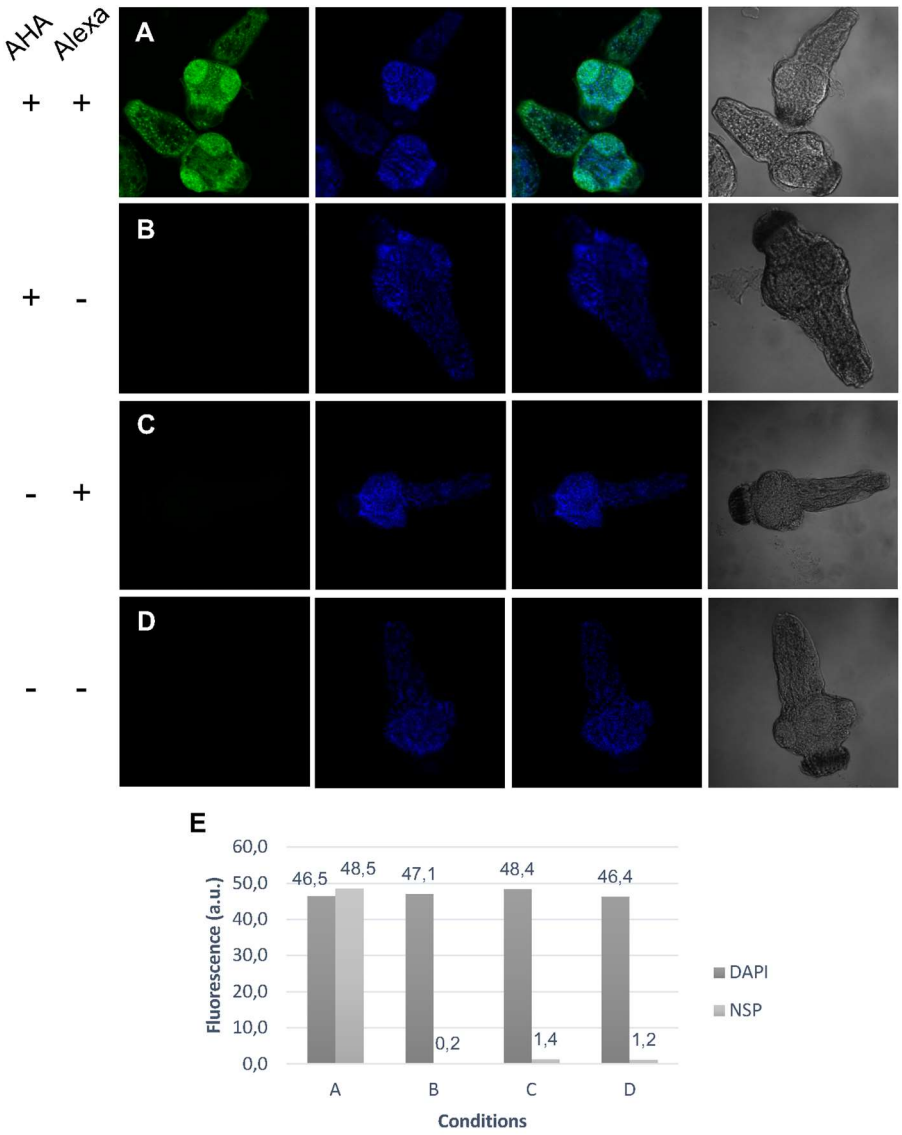


**Fig 2. *In toto* visualization of proteins synthesized by *E. granulosus* PSCs induced to strobilar development.** (A) NSPs were specifically visualized in the presence of AHA and Alexa Fluor 488 Alkyne. The other images represent the DAPI nuclei staining, the bright field merges of DAPI and Alexa Fluor 488 antibody staining and the bright field images. (B) AHA+/Alexa-, (C) AHA-/Alexa+ and (D) AHA-/Alexa- did not show significant fluorescence or autofluorescence (400x). (E) The quantification of NSP (Alexa Fluor 488) and nucleic acid regions (DAPI) fluorescence. a.u., arbitrary units.

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t-test to find the differentially expressed proteins. 75 proteins were considered to be differentially expressed, with 34 considered to be more highly expressed in SSD and 41 more highly expressed in NSD (Table 1).

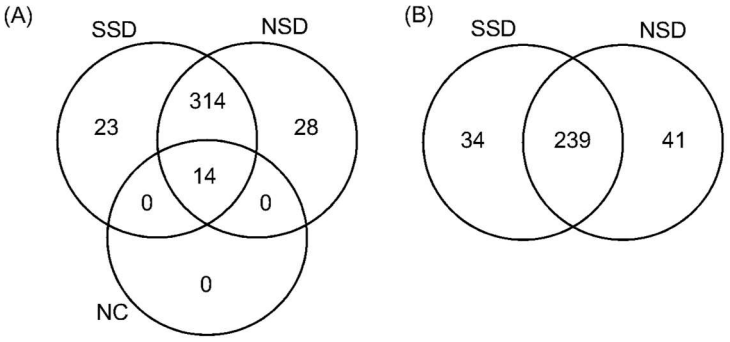


**Fig 3. Proteins identified in *E. granulosus* PSCs after the induction of strobilar development.** Venn diagram showing the identified proteins in the AHA and control samples. (A) Exclusive proteins identified in SSD and CSD. (B) Differentially expressed proteins.

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In SSD, several proteins are involved in metabolic reactions (Fig 4) and may indicate how the parasite obtains energy during its development into the adult worm. A feasible metabolic pathway for *E. granulosus* involves phosphoenolpyruvate, which is carboxylated to give oxaloacetate. Oxaloacetate is reduced to malate, which is then either oxidatively decarboxylated to pyruvate or reduced to succinate. Moreover, pyruvate can be converted into acetyl-CoA, which can participate in the tricarboxylic acid cycle or be converted into acetate.

A functional annotation of the NSPs is presented in Table 1 (data summarized in Fig 5A). The most representative terms in the SSD were related to post-translational modification, protein turnover, and chaperones (O, 30%), energy production and conversion (C, 19%), cytoskeleton (Z, 11%), intracellular trafficking, secretion, and vesicular transport (U, 9%) and transcription (K, 7%). In the NSD condition, the most representative functions were cytoskeleton (Z, 20%), signal transduction mechanisms (T, 17%), post-translational modification, protein turnover, and chaperones (O, 15%) and RNA processing and modification (A, 10%). A hierarchical clustering of the up-regulated proteins listed in Table 1 was performed using the Z-score calculation on NSAF, and the results were represented as a heat map (Fig 5B). Two main clusters of the NSPs during the strobilar stimuli showing high (red) or low (green) expression.

## Discussion

Techniques that allow for the selective labeling of molecular targets are powerful tools for understanding the molecular pathways involved in the strobilation process and the identification of key proteins that are activated in response to specific stimuli. The high specificity of AHA-labeling has been proven to be non-toxic as it does not alter the global protein synthesis rates or cause significant protein misfolding or degradation [16, 19, 20, 31]. Furthermore, AHA is incorporated exclusively into NSPs without interfering with preexisting proteins.

Ethical and practical difficulties in undertaking *in vivo* studies bring up the necessity to develop *in vitro* systems. Thus, we have successfully obtained an *in vitro* model of *E. granulosus* PSCs undergoing strobilar development under low potential sources of Met and conditions that facilitate the efficient incorporation of AHA. The genomic data for *E. granulosus* were also analyzed and confirm the feasibility of AHA labeling because proteins with non-terminal Met residues are required for this purpose (S1 Text). Another factor contributing to the success of

**Table 1. Differentially expressed proteins.**

| Functional annotation[a] | Identified proteins[b,c] | Accession Number | NSAF SSD[d] | NSAF NSD[e] | P-value | Fold change |
|---|---|---|---|---|---|---|
| | SSD proteins | | | | | |
| Amino acid transport and metabolism | Glutamate dehydrogenase mitochondrial | EgrG_000604400.1 | 0,00218163 | 0 | - | - |
| | 2 amino 3 ketobutyrate coenzyme A ligase | EgrG_000107200.1 | 0,00500288 | 0 | - | - |
| Cytoskeleton | Myosin regulatory light chain A, smooth adductor muscle | gi\|576695936 | 0,14420750 | 0,11952750 | 0,0027 | 1,2 |
| | Actin cytoplasmic type 5 | EgrG_000190400.1 | 0,01251020 | 0,00709483 | 0,034 | 1,8 |
| | Myosin heavy chain, striated muscle | gi\|576698220 | 0,02525800 | 0,01340450 | 0,0017 | 1,9 |
| | Dynein light chain 1, cytoplasmic | gi\|576696895 | 0,13540500 | 0,06898675 | 0,0043 | 2,0 |
| | Myosin regulatory light chain 2 smooth muscle | EgrG_000041600.1 | 0,02763825 | 0,01373125 | 0,049 | 2,0 |
| | Actin cytoplasmic A3 | EgrG_000406900.1 | 0,01701225 | 0,00795388 | 0,0055 | 2,1 |
| Energy production and conversion | ATP synthase subunit beta mitochondrial | EgrG_000752000.1 | 0,00210028 | 0 | - | - |
| | NADP dependent malic enzyme | EgrG_001145700.1 | 0,00237255 | 0 | - | - |
| | Pyruvate dehydrogenase E1 component subunit | EgrG_000590700.1 | 0,00304668 | 0 | - | - |
| | NADH cytochrome b5 reductase 3 | EgrG_000865900.1 | 0,00339048 | 0 | - | - |
| | Aconitate hydratase mitochondrial | EgrG_000158240.1 | 0,00365168 | 0 | - | - |
| | Succinate dehydrogenase ubiquinone | EgrG_000422600.1 | 0,00774378 | 0 | - | - |
| | Pyruvate dehydrogenase | EgrG_000956200.1 | 0,00862753 | 0 | - | - |
| | Cytosolic malate dehydrogenase | EgrG_000417100.1 | 0,05691600 | 0,03523775 | 0,036 | 1,6 |
| | Succinate dehydrogenase (ubiquinone) iron sulfur | EgrG_000416100.1 | 0,04523500 | 0,02794375 | 0,00045 | 1,6 |
| | Phosphoenolpyruvate carboxykinase [GTP] | gi\|576694081 | 0,07943825 | 0,04717400 | < 0,00010 | 1,7 |
| | Citrate synthase | EgrG_001028500.1 | 0,02500050 | 0,01324200 | 0,00059 | 1,9 |
| Function unknown | Hypothetical protein | gi\|576695609 | 0,00378138 | 0 | - | - |
| | RNA binding protein EWS | EgrG_001020700.1 | 0,01534125 | 0,00991710 | 0,011 | 1,5 |
| | Expressed conserved protein | EgrG_000696700.1 | 0,00851018 | 0,00211125 | 0,015 | 4,0 |
| | Hypothetical protein | gi\|576692667 | 0,01587075 | 0,00369325 | 0,023 | 4,3 |
| Intracellular trafficking, secretion, and vesicular transport | Charged multivesicular body protein | gi\|576693582 | 0,01026600 | 0 | - | - |
| | Charged multivesicular body protein 4B | EgrG_001133200.1 | 0,02666625 | 0,01683500 | 0,041 | 1,6 |
| | Clathrin light chain | EgrG_000925600.1 | 0,01228048 | 0,00764348 | 0,025 | 1,6 |
| | Charged multivesicular body protein 5 | EgrG_001063800.1 | 0,01977925 | 0,00958983 | 0,011 | 2,1 |
| | Charged multivesicular body protein 3 | EgrG_000238400.1 | 0,02452800 | 0,00300075 | 0,0042 | 8,2 |
| Nucleotide transport and metabolism | Nucleoside diphosphate kinase A 2 | gi\|576695995 | 0,00894200 | 0 | - | - |
| | Dihydropyrimidinase | EgrG_000953400.1 | 0,01286375 | 0,00754355 | 0,005 | 1,7 |
| Post-translational modification, protein turnover, and chaperones | Heat shock 70 kDa protein 4 | EgrG_000938600.1 | 0,00130175 | 0 | - | - |
| | 26S proteasome regulatory subunit T3 | EgrG_000223000.1 | 0,00212315 | 0 | - | - |
| | T-complex protein 1 subunit epsilon | gi\|576697091 | 0,00235945 | 0 | - | - |
| | Calreticulin | gi\|576693417 | 0,00287313 | 0 | - | - |

(*Continued*)

**Table 1.** (*Continued*)

| Functional annotation[a] | Identified proteins[b,c] | Accession Number | NSAF SSD[d] | NSAF NSD[e] | P-value | Fold change |
|---|---|---|---|---|---|---|
| | Activator of 90 kDa heat shock protein ATPase | EgrG_000241900.1 | 0,00451208 | 0 | - | - |
| | T-complex protein 1 subunit zeta | gi\|576693013 | 0,00625755 | 0 | - | - |
| | 26S proteasome regulatory subunit N11 | EgrG_001166800.1 | 0,00680358 | 0 | - | - |
| | Heat shock protein | gi\|576694604 | 0,00444963 | 0,00395170 | 0,0058 | 1,1 |
| | Transitional endoplasmic reticulum ATPase | EgrG_000471600.1 | 0,03403750 | 0,02595500 | 0,024 | 1,3 |
| | Ubiquitin conjugating enzyme | EgrG_000616800.1 | 0,04567950 | 0,03198050 | 0,0043 | 1,4 |
| | Calnexin | EgrG_000875100.1 | 0,00900948 | 0,00611455 | 0,027 | 1,5 |
| | WAP, Kazal, immunoglobulin, Kunitz and NTR domain-containing protein 2 | gi\|576694291 | 0,03321825 | 0,02210675 | 0,042 | 1,5 |
| | Major egg antigen p40 | EgrG_000212700.1 | 0,06261775 | 0,03261750 | 0,00025 | 1,9 |
| | Ubiquilin-1 | gi\|576694418 | 0,01723425 | 0,00737003 | 0,00038 | 2,3 |
| | Heat shock protein 60 | EgrG_001190900.1 | 0,11921750 | 0,04692450 | < 0,00010 | 2,5 |
| | Protein AHNAK2 | EgrG_000760400.1 | 0,01505950 | 0,00299023 | < 0,00010 | 5,0 |
| | T complex protein 1 subunit gamma | EgrG_000872100.1 | 0,00943840 | 0,00080528 | 0,0044 | 11,7 |
| RNA processing and modification | RNA recognition motif RRM domain containing protein | EgrG_000264300.1 | 0,00222925 | 0 | - | - |
| | Spliceosome RNA helicase BAT1 | EgrG_000546900.1 | 0,00705580 | 0,00101435 | 0,0082 | 7,0 |
| Signal transduction mechanisms | Calcium binding protein P22 | EgrG_000447500.1 | 0,04368925 | 0,02742450 | 0,038 | 1,6 |
| Transcription | Serrate RNA effector molecule | gi\|576694739 | 0,00042201 | 0 | - | - |
| | SNW domain-containing protein | gi\|576692125 | 0,00238970 | 0 | - | - |
| | Transcription elongation regulator 1 | EgrG_000862700.1 | 0,00334500 | 0,00144693 | 0,0082 | 2,3 |
| | Elongation factor 1 delta | EgrG_000517100.1 | 0,01624900 | 0,00681798 | < 0,00010 | 2,4 |
| Translation, ribosomal structure and biogenesis | Aminoacyl tRNA synthase complex interacting | EgrG_001100200.1 | 0,00423773 | 0 | - | - |
| | 40S ribosomal protein AS | EgrG_000720100.1 | 0,02209325 | 0,00692395 | 0,00023 | 3,2 |
| | Eukaryotic translation initiation factor 3 | EgrG_000068000.1 | 0,00730010 | 0,00151090 | 0,018 | 4,8 |
| | **NSD proteins** | | | | | |
| Carbohydrate transport and metabolism | Triosephosphate isomerase | EgrG_000416400.1 | 0,01662075 | 0,02560950 | 0,044 | 1,5 |
| | Fructose 16 bisphosphate aldolase | EgrG_000905600.1 | 0,03377200 | 0,05451625 | 0,00016 | 1,6 |
| Cell cycle control, cell division, chromosome partitioning | Rab11 family-interacting protein | gi\|576698836 | 0 | 0,0016588 | - | - |
| | Structural maintenance of chromosomes protein 2 | EgrG_000602100.1 | 0,0073003 | 0,0101055 | 0,0069 | 1,4 |
| | Translationally controlled tumor protein | EgrG_000058000.1 | 0,0060565 | 0,0223265 | 0,012 | 3,7 |
| Cell wall/membrane/envelope biogenesis | Ankyrin-3 | gi\|576691831 | 0 | 0,00062894 | - | - |
| Cytoskeleton | Protein kinase C and casein kinase substrate in | EgrG_001090800.1 | 0 | 0,00113730 | - | - |
| | Ezrin | EgrG_000517000.1 | 0 | 0,00118645 | - | - |
| | Troponin i | EgrG_000734700.1 | 0 | 0,00336850 | - | - |
| | Microtubule associated protein RP:EB family | EgrG_000637300.1 | 0 | 0,00603648 | - | - |
| | Dynein light chain | EgrG_000071300.1 | 0 | 0,02840825 | - | - |
| | Myosin essential light chain, striated adductor muscle | gi\|576700259 | 0,11973250 | 0,15936500 | 0,00025 | 1,3 |
| | Spectrin alpha chain | gi\|576696380 | 0,00739963 | 0,00999265 | 0,0081 | 1,4 |

(*Continued*)

**Table 1.** (*Continued*)

| Functional annotation[a] | Identified proteins[b,c] | Accession Number | NSAF SSD[d] | NSAF NSD[e] | P-value | Fold change |
|---|---|---|---|---|---|---|
| | Myophilin | gi\|576693412 | 0,01986200 | 0,02702125 | 0,03 | 1,4 |
| | Tubulin alpha-1C chain | gi\|576701165 | 0,01033978 | 0,01517000 | 0,028 | 1,5 |
| | Tropomyosin-2 | gi\|576693326 | 0,00569973 | 0,00891180 | 0,035 | 1,6 |
| | Actin modulator protein | EgrG_000882500.1 | 0,01606350 | 0,02763525 | 0,0032 | 1,7 |
| | Gamma aminobutyric acid receptor associated | EgrG_001158000.1 | 0,01022620 | 0,02080425 | 0,0037 | 2,0 |
| | Hematopoietic lineage cell specific protein | EgrG_000936900.1 | 0,00072758 | 0,00487913 | 0,0095 | 6,7 |
| | Cofilin/actin-depolymerizing factor | gi\|576697710 | 0,00215878 | 0,02190575 | 0,0012 | 10,1 |
| Function unknown | Expressed conserved protein | EgrG_000203800.1 | 0 | 0,00223970 | - | - |
| | Hypothetical protein | gi\|576699770 | 0 | 0,00279715 | - | - |
| | Guanine nucleotide binding protein subunit | EgrG_000200300.1 | 0 | 0,00626868 | - | - |
| | Cystatin B stefin B | EgrG_000159200.1 | 0 | 0,01504050 | - | - |
| | Expressed conserved protein | EgrG_001061900.1 | 0,00958830 | 0,01356800 | 0,03 | 1,4 |
| | Hypothetical protein | gi\|576700828 | 0,00681093 | 0,01128425 | 0,0049 | 1,7 |
| | Sj Ts4 protein | EgrG_000393000.1 | 0,01064943 | 0,02080450 | 0,0028 | 2,0 |
| | Hypothetical protein | gi\|576692345 | 0,00112804 | 0,00235835 | 0,0058 | 2,1 |
| | Programmed cell death 6 interacting protein | EgrG_000997550.1 | 0,00064300 | 0,00363378 | 0,04 | 5,7 |
| | Hypothetical protein | gi\|576690197 | 0,00036507 | 0,00254528 | 0,0052 | 7,0 |
| Inorganic ion transport and metabolism | Na:K ATPase alpha subunit | EgrG_000342600.1 | 0,00232473 | 0,00436733 | 0,018 | 1,9 |
| Intracellular trafficking, secretion, and vesicular transport | Sorting nexin | EgrG_000922200.1 | 0 | 0,01089525 | - | - |
| | Annexin A6 | gi\|576697441 | 0,00147718 | 0,00481598 | 0,017 | 3,3 |
| Post-translational modification, protein turnover, and chaperones | Ubiquitin supergroup | EgrG_001180300.1 | 0 | 0,01742975 | - | - |
| | Heat shock protein 71 kDa protein | EgrG_001085100.1 | 0,02868700 | 0,03494425 | 0,048 | 1,2 |
| | Stress induced phosphoprotein 1 | EgrG_000264900.1 | 0,03160575 | 0,03906600 | 0,032 | 1,2 |
| | Thioredoxin peroxidase | EgrG_000791700.1 | 0,05674150 | 0,07183575 | 0,0053 | 1,3 |
| | Dnaj subfamily A | EgrG_000101800.1 | 0,03477350 | 0,04575175 | 0,0057 | 1,3 |
| | Dnaj subfamily B | EgrG_000614200.1 | 0,03305725 | 0,04662200 | 0,0029 | 1,4 |
| | Calreticulin | gi\|576693418 | 0,00577333 | 0,00862368 | 0,033 | 1,5 |
| | Calpain | EgrG_000719700.1 | 0,00653558 | 0,01014048 | 0,017 | 1,6 |
| | Ubiquitin-conjugating enzyme E2 L3 | gi\|576700841 | 0,00335100 | 0,01493200 | 0,031 | 4,5 |
| | Tumor protein D52 | EgrG_000949820.1 | 0,00238700 | 0,01167500 | 0,0081 | 4,9 |
| Replication, recombination and repair | Phosphatase 2A inhibitor I2PP2A | EgrG_000465500.1 | 0,02583550 | 0,03972525 | 0,0014 | 1,5 |
| RNA processing and modification | RNA binding protein fox 1 3 | EgrG_000063400.1 | 0 | 0,00132858 | - | - |
| | ATP dependent RNA helicase DDXx | EgrG_000098400.1 | 0 | 0,00231058 | - | - |
| | U2 small nuclear RNA auxiliary factor 2 | EgrG_000625400.1 | 0 | 0,00292313 | - | - |
| | Polyadenylate binding protein | EgrG_000689000.1 | 0,00971535 | 0,01186025 | 0,036 | 1,2 |
| | Heterogeneous nuclear ribonucleoprotein A1 | EgrG_000807100.1 | 0,03108225 | 0,04304250 | 0,00081 | 1,4 |

(*Continued*)

**Table 1.** (*Continued*)

| Functional annotation[a] | Identified proteins[b,c] | Accession Number | NSAF SSD[d] | NSAF NSD[e] | P-value | Fold change |
|---|---|---|---|---|---|---|
| | Fragile X mental retardation syndrome-related protein | gi\|576697665 | 0,00311460 | 0,00595435 | 0,026 | 1,9 |
| | Heterogeneous nuclear ribonucleoprotein K | gi\|576694356 | 0,00188858 | 0,00516005 | 0,0063 | 2,7 |
| Secondary metabolites biosynthesis, transport, and catabolism | ES1 protein mitochondrial | EgrG_000999800.1 | 0 | 0,00349188 | - | - |
| Signal transduction mechanisms | SH2 motif | EgrG_000343500.1 | 0 | 0,00097938 | - | - |
| | Tyrosine protein kinase otk | EgrG_000212300.1 | 0 | 0,00210218 | - | - |
| | Nuclear migration protein nudc | EgrG_000463400.1 | 0 | 0,00241293 | - | - |
| | PDZ domain containing protein GIPC3 | EgrG_000190700.1 | 0 | 0,00396823 | - | - |
| | Na:H exchange regulatory cofactor NHE RF2 | EgrG_000743800.1 | 0 | 0,00443958 | - | - |
| | Serine:threonine protein phosphatase PP1 gamma | EgrG_000779500.1 | 0 | 0,00607725 | - | - |
| | Transforming protein RhoA | EgrG_000246600.1 | 0 | 0,00812860 | - | - |
| | Neuronal calcium sensor | EgrG_000186300.1 | 0 | 0,01437175 | - | - |
| | Titin | EgrG_000610500.1 | 0,00034956 | 0,00063859 | 0,009 | 1,8 |
| | Universal stress protein | EgrG_000873800.1 | 0,00811793 | 0,02731575 | 0,00055 | 3,4 |
| | Arfaptin 2 | EgrG_000192300.1 | 0,00156715 | 0,00686460 | 0,039 | 4,4 |
| | Endophilin B2 | EgrG_000060900.1 | 0,00288233 | 0,01315925 | 0,0056 | 4,6 |
| Transcription | Transcriptional repressor P66-beta | gi\|576694405 | 0 | 0,00156535 | - | - |
| | Transcription factor AP 4 | EgrG_000768400.1 | 0 | 0,00621390 | - | - |
| | Actin-depolymerizing factor 2 | gi\|576697711 | 0,00135023 | 0,00318298 | 0,03 | 2,4 |
| Translation, ribosomal structure and biogenesis | 40S ribosomal protein S28 | EgrG_001150800.1 | 0 | 0,03077775 | - | - |
| | Ribosomal protein LP1 | EgrG_001195100.1 | 0,03494200 | 0,04921800 | 0,015 | 1,4 |

The proteins that were identified as exclusive in at least two replicates or the proteins that were differentially expressed with significant T-Test values ($p \leq 0.05$) are presented.

[a]Functional classification determined by eggNOG.

[b]Protein accession numbers according to GeneDB (www.genedb.org/).

[c]Protein accession numbers according to NCBI (www.ncbi.nlm.nih.gov/).

[d]Average NSAF for SSD replicates.

[e]Average NSAF for NSD replicates.

doi:10.1371/journal.pntd.0004085.t001

AHA-labeling is that the genome of this parasite showed no machinery for the endogenous synthesis of Met [6].

SDS-PAGE and confocal microscopy were useful in detecting the incorporation of AHA. The confocal microscopy showed a possible correlation between the NSPs and PSCs suckers. In *Mesocestoides corti*, the apical massif is a polynucleated cell mass that differentiates into several cell types [32]. This structure is a part of the tegmental syncytium and is located at the top of the scolex, next to the suckers. Although studies using DNA labeling in *E. granulosus* have not demonstrated the existence of a proliferation site in this region [33], the possible correlation between the NSPs and the suckers may correspond to an increased protein synthesis site.

The expression pattern identified by proteomic analysis of NSPs from PSC without stimuli for strobilar development revealed proteins involved in basic cellular functions, such as

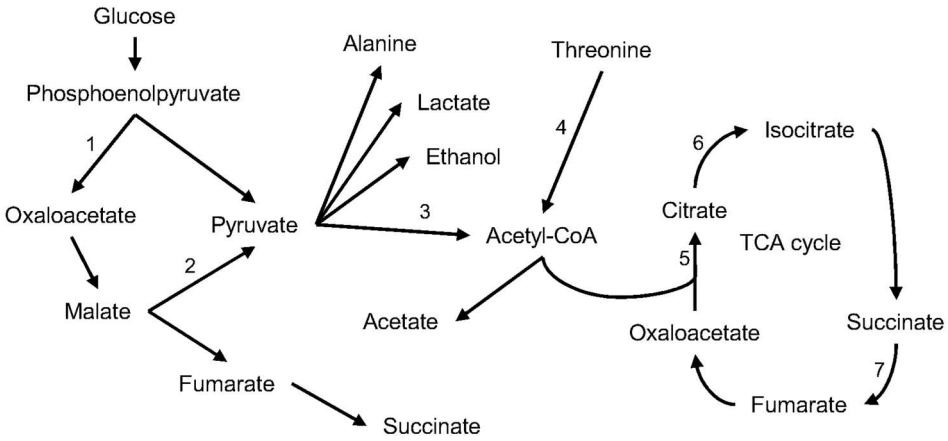


**Fig 4. Metabolism in *E. granulosus* PSCs after induction of the strobilar stage.** A representative schematic of the metabolic pathways that may be active during the strobilar development of PSCs. These reactions comprise the degradation of carbohydrates to phosphoenolpyruvate and the production of pyruvate and succinate.1) Phosphoenolpyruvate carboxykinase; 2) NADP-dependent malic enzyme; 3) Pyruvate dehydrogenase; 4) 2 amino 3 ketobutyrate coenzyme A ligase; 5) Citrate synthase; 6) Aconitate hydratase mitochondrial; and 7) Succinate dehydrogenase ubiquinone.

doi:10.1371/journal.pntd.0004085.g004

metabolic processes, regulation of biological processes and cellular component organization. In this sample, we have identified proteins related to transcription, translation and cytoskeletal.

In contrast, proteomic analysis of NSPs from SSD samples indicated changes in parasite metabolism and this has been reported with development [34–36] In more advanced stages of worm development, there is a shift from cytosolic to mitochondrial metabolism, which tends to produce more acetate and succinate, two end products with a higher energy yield than lactate [36–38]. Thereby, changes in energy-producing pathways associated with maturation may be essential for both the correct progression of parasite life cycle as well as survival.

We found a SNW domain-containing protein that is a member of the SNW gene family. Human SNW encodes a transcription coactivator that can interact with vitamin D receptor (VDR) and retinoid X receptor (RXR) [39]. It is believed that VDR and RXR may play key roles in stimulating PSCs to develop into adult worms [6, 40]. The binding and activation of these receptors by bile acid salts regulates the expression of genes involved in differentiation, development, homeostasis and metabolism. Therefore, finding an SNW protein may indicate the presence of an active state of VDR/RXR in SSD, which is plausible given these findings.

We also found three charged multivesicular body proteins that are components of the endosomal sorting complex required for transport III (ESCRT-III) [41–43]. ESCRT-III participates in the degradation of the surface receptor proteins, the formation of endocytic multivesicular bodies and the down-regulation of several signaling pathways. We also identified a clathrin light chain, a subunit of clathrin that participates in several membrane traffic pathways [44–46]. Extracellular vesicles are derived from the multivesicular body and act in host/parasite relationships and cell–cell signaling [47, 48]. Although this is a preliminary result for *E. granulosus*, it is encouraging to find proteins related to these functions. This cell-cell communication via exosome-like vesicles has been related to sexual differentiation, survival and population density [49].

Interestingly, a comparison of the SSD up-regulated proteins showed no apparent correlation with the previously published RNAseq data [6]. However, RNAseq data includes only expression profiles from either pepsin-activated PSCs or adult worms collected from dogs,

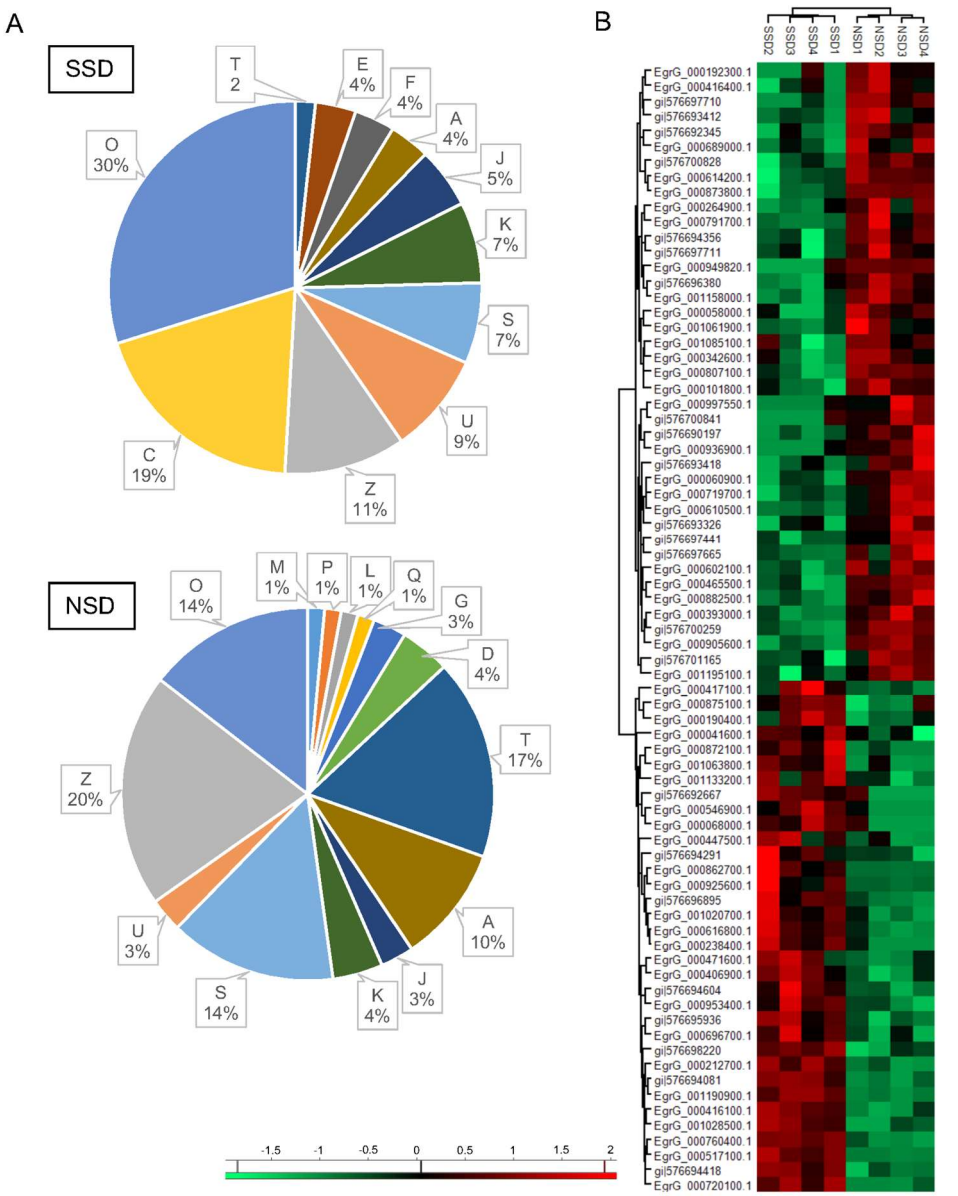


**Fig 5. Comparative analysis of NSPs from *E. granulosus* PSCs after the induction of strobilar development.** (A) Functional categories of total identified NSPs. Percentages of identified proteins in each functional category are indicated. (O) Post-translational modification, protein turnover, and chaperones; (C) Energy production and conversion; (Z) Cytoskeleton; (U) Intracellular trafficking, secretion, and vesicular transport; (S) Function unknown; (K) Transcription; (J) Translation, ribosomal structure and biogenesis; (A) RNA processing and modification; (F) Nucleotide transport and metabolism; (E) Amino acid transport and metabolism; (T) Signal transduction mechanisms; (D) Cell cycle control, cell division, chromosome partitioning; (G) Carbohydrate transport and metabolism; (Q) Secondary metabolites biosynthesis, transport, and catabolism; (L) Replication, recombination and repair; (P) Inorganic ion transport and metabolism; (M) Cell wall/membrane/envelope biogenesis. The distribution of level 3 biological processes for SSD and NSD-exclusive and up-regulated proteins. (B) A heat map from NSPs with high (red) or low (green) expression levels between the SSD and NSD groups.

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with no data available for the transition between these two stages. Therefore, we believe that this only reinforces the importance of our experimental approach in the attempt to identify early molecular events that are triggered by a developmental stimulus.

This is the first report of an efficient labeling and identification of NSPs with AHA in flatworms, which provides an interesting tool for use in the search for regulatory molecules in *E. granulosus* and other parasitic organisms. The temporally controlled and context-dependent labelling of synthesized proteins allow the association between molecular changes and the processes occurring during induction of strobilation. Whereas the steps of induction between different stages play a central role to the correct development of the parasite, the knowledge of such processes can have great value to the improvement of new disease control strategies. Although there is vaccine for the intermediate host [50], the WHO recommends that other stages should also be targeted for intervention [1], which would make more efficient control. Still, considering that regulatory processes may be conserved among different helminths, the results obtained here can serve as a starting point for control studies of other parasites.

## Supporting Information

**S1 Fig. *In vitro* induction of strobilar development in *E. granulosus* PSCs.** PSCs after pepsin treatment (A) and after 3 days in culture without stimuli for strobilar development (B). After one week, NSD worms present a small number of calcareous corpuscles (C). PCSs after 3 days in complete biphasic medium (D). After 5 days, calcareous corpuscles are much reduced and excretory canals (e) become evident (E) and, after one week, posterior excretory bladder (bl) is also visible (F) Magnification, 100x (A, B and D) and 200x (C, E and F).
(TIF)

**S2 Fig. AHA is incorporated in proteins synthesized by *E. granulosus* PSCs.** After incubation with 0–100 μM AHA for 12–72 h, the biotin-labeled AHA-containing NSPs were detected by immunoblot (A) and quantified (B) by comparing the intensities of the sample dots and the biotinylated Bovine Serum Albumin standard dots.
(TIF)

**S1 Table. NSPs identified in the *E. granulosus* PSCs.** The complete list of peptides and proteins normalized by NSAF is shown. The qualitative profiles for Excl SSD or Excl NSD (protein exclusive to SSD or NSD, respectively), SSD-1R or NSD-1R (proteins identified in just one replicate of SSD or NSD, respectively), or NC-excluded (proteins identified in the negative control and excluded from analysis) or the quantitative profiles for SSD (SSD high, NSD low) or NSD (SSD low, NSD high) with their respective T-Test values are also shown.[a]Protein accession numbers according to GeneDB (www.genedb.org/).[b]Protein accession numbers according to NCBI (www.ncbi.nlm.nih.gov/).
(XLSX)

**S1 Text. Supplemental experiments.** Additional methods and results used in genomic data analysis, PCS cultivation and dot blot assays.
(DOCX)

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## Author Contributions

Conceived and designed the experiments: JAD KMM HBF AZ. Performed the experiments: JAD KMM HM HBF. Analyzed the data: JAD KMM HBF AZ. Contributed reagents/materials/analysis tools: KMM HM JRB HBF AZ. Wrote the paper: JAD KMM HM HBF AZ.

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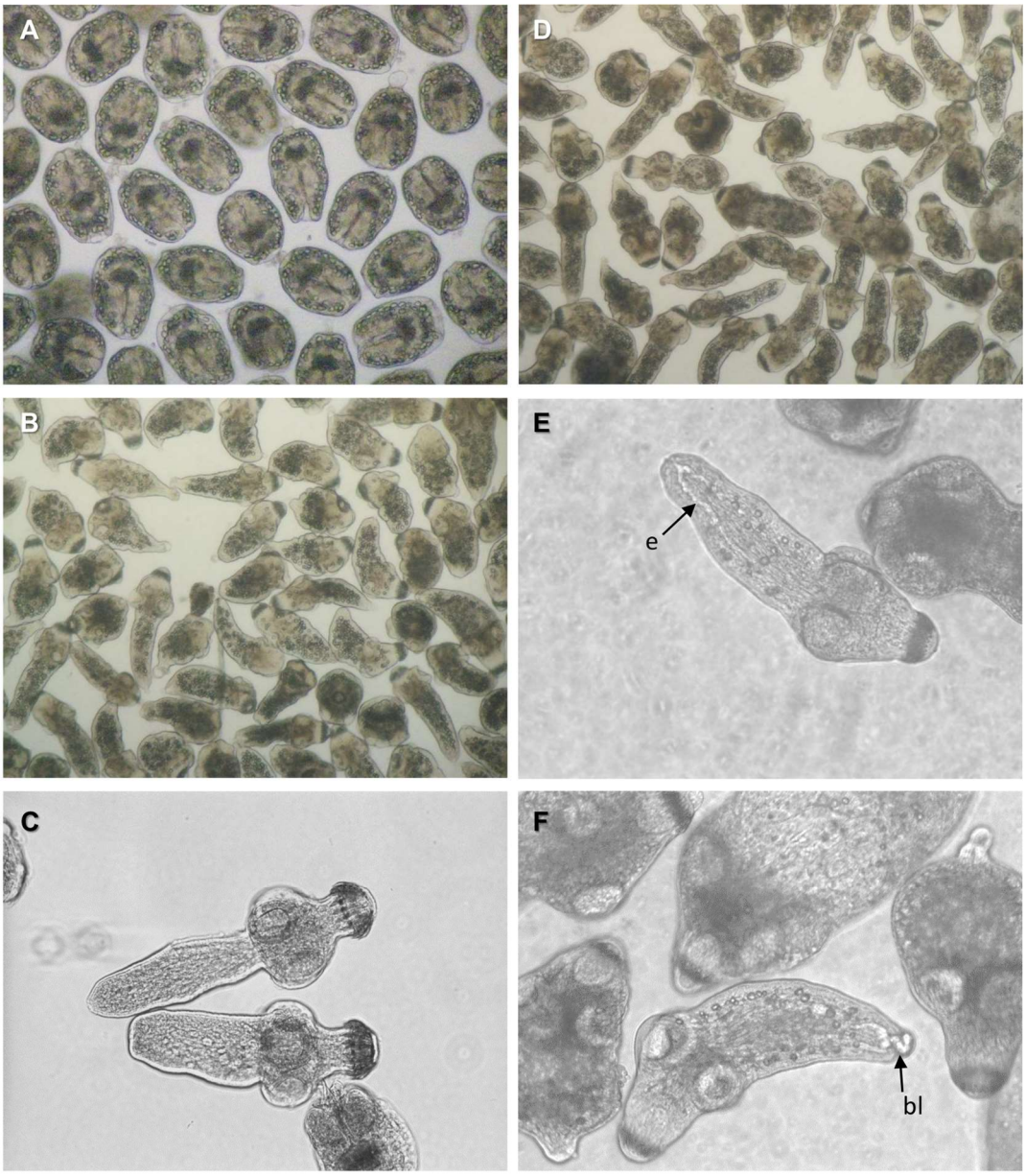
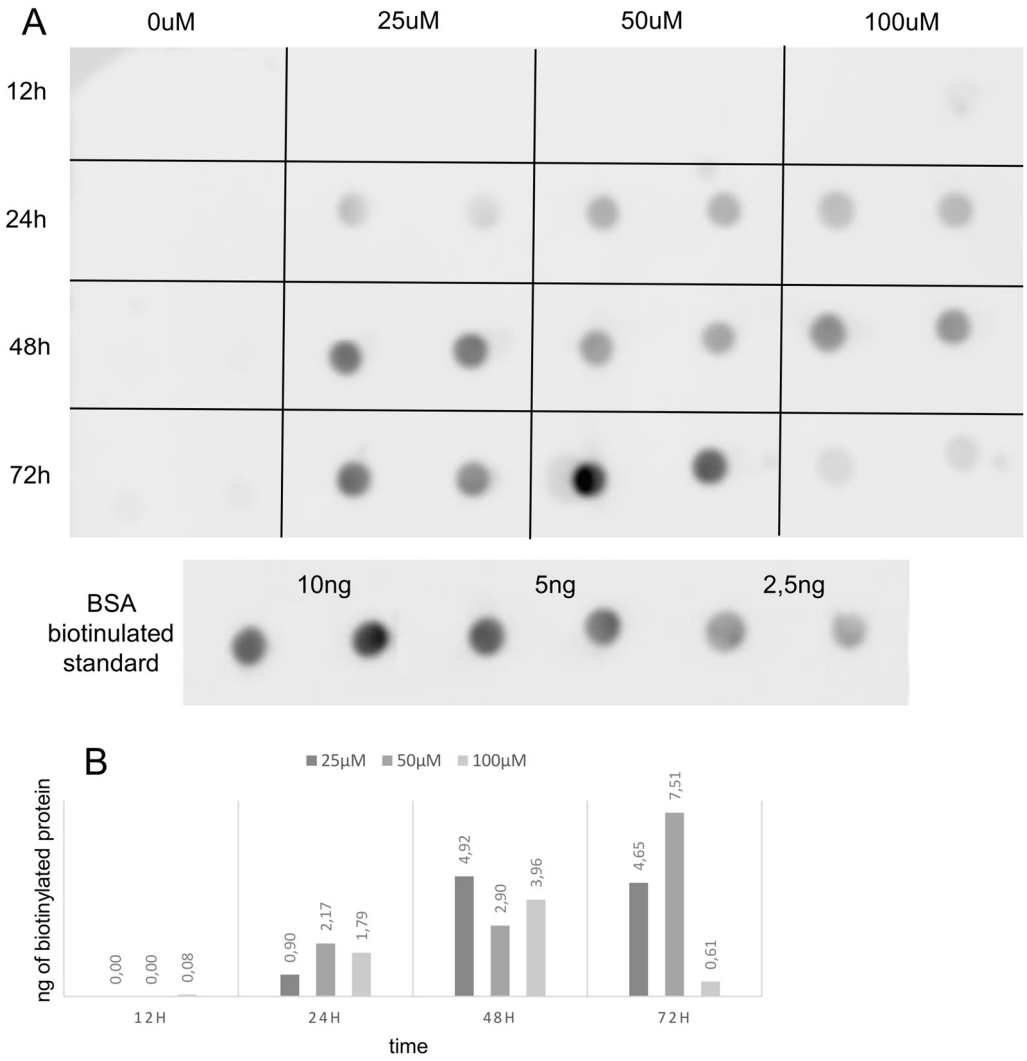



**Supplementary Figure 1. *In vitro* induction of strobilar development in *E. granulosus* PSCs.**

PSCs after pepsin treatment (A) and after 3 days in culture without stimuli for strobilar development (B). After one week, NSD worms present a small number of calcareous corpuscles (C). PCSs after 3 days in complete biphasic medium (D). After 5 days, calcareous corpuscles are much reduced and excretory canals (e) become evident (E) and, after one week, posterior excretory bladder (bl) is also visible (F) Magnification, 100x (A, B and D) and 200x (C, E and F).




**Supplementary Figure 2. AHA is incorporated in proteins synthesized by *E. granulosus* PSCs.**

After incubation with 0–100 µM AHA for 12−72 h, the biotin-labeled AHA-containing NSPs were detected by immunoblot (A) and quantified (B) by comparing the intensities of the sample dots and the biotinylated Bovine Serum Albumin standard dots.

## **4. Capítulo II** - Transcriptomic analysis of the early strobilar development of *Echinococcus granulosus*

João Antonio Debarba, Karina Mariante Monteiro, Alexandra Lehmkuhl Gerber, Ana Tereza Ribeiro de Vasconcelos, Arnaldo Zaha

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**Transcriptomic analysis of the early strobilar development of *Echinococcus granulosus***

João Antonio Debarba[1], Karina Mariante Monteiro[1,2], Alexandra Lehmkuhl Gerber[3], Ana Tereza Ribeiro de Vasconcelos[3], Arnaldo Zaha[1,2]*

1 Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

2 Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

3 Laboratório Nacional de Computação Científica, Petrópolis, Rio de Janeiro, Brazil

*Corresponding author:

E-mail: zaha@cbiot.ufrgs.br (AZ)

**Abstract**

**Background:** *Echinococcus granulosus* has a complex life cycle involving two mammalian hosts. The transition from one host to another is accompanied by changes in gene expression, and the transcriptional events that underlie these processes have not yet been fully characterized.

**Results:** In this study, RNA-seq is used to compare the transcription profiles of four time samples of *E. granulosus* protoscoleces *in vitro* induced to strobilar development. We identified 818 differentially expressed genes, which were divided into eight expression clusters formed over the entire 24 hours time course and indicated different transcriptional patterns. An enrichment of gene transcripts with molecular functions of signal transduction, enzymes and protein modifications was observed with progression of development.

**Conclusion:** This transcriptomic study provides insight for understanding the complex life cycle of *E. granulosus* and contributes for searching for the key genes correlating with the strobilar development, providing interesting hints for further studies.

**Keywords:** *Echinococcus granulosus*, RNA-seq, transcriptome, strobilar development.

## Background

Echinococcosis is a zoonotic parasitic infection caused by tapeworms of the genus *Echinococcus* and considered as one of the 17 neglected tropical diseases prioritized by the World Health Organization [1,2]. The two most important forms of the disease are cystic echinococcosis (hydatidosis) and alveolar echinococcosis, caused by infection with *Echinococcus granulosus* and *Echinococcus multilocularis,* respectively. *Echinococcus* has a two-host life cycle with the larval stage growing in the tissues of an intermediate host (large variety of non-carnivorous species) and the adult stage living in the intestine of a definitive host (few species of carnivores) [3,4].

*E. granulosus* larvae, also called metacestode, is a fluid-filled vesicular cyst containing many infectious protoscoleces, the pre-adult forms of the parasite. *In vitro* studies demonstrated that protoscoleces have an unusual potential of being able to differentiate in two different directions, depending on the environmental conditions provided. In the intermediate host, upon hydatid cyst rupture, protoscoleces released in the body cavity differentiate in a cystic direction (secondary hydatid cysts). In contrast, protoscoleces ingested by a dog and exposed to the gut environment, sexually differentiate in a strobilar direction to form fully developed, segmented adult tapeworms [4–7].

The strobilar development is directly influenced by the host-parasite relationship and configure a key point for the life cycle. Initially, the protoscolex remains quiescent and invaginated within the cyst until it receives the correct stimuli for the strobilation upon ingestion by a definitive host [7,8]. The nature of these stimuli is not fully known, but it is believed that chewing and the proteolytic enzymes like pepsin have a considerable role in this process, as well as temperature and the presence of bile salts. In addition, histological studies have already demonstrated that parasite binding or contact with a substrate similar to that found on the surface of the canine gut also constitutes an important stimulus for strobilation [9–11]. These observations resulted

in the elaboration of strategies for *in vitro* culture of *Echinococcus* protoscoleces in order to provide the necessary physiological conditions for parasite strobilar development (summarized in Smyth, 1990).

In the molecular aspects, genome and transcriptome studies for *E. granulosus* [13–15] and *E. multilocularis* [14,16] identified differentially expressed genes between parasite life stages. Furthermore, considerable losses and gains of genes that may be associated with adaptations to parasitism were identified. In *E. granulosus*, crucial genes or even entire pathways of *de novo* synthesis of fatty acids, cholesterol, pyrimidines, purines and most amino acids are absent. Thus, *E. granulosus* relies on the host for obtaining these nutrients. Specifically in relation to strobilar development, it is known that bile acids have a crucial role in the differentiation of protoscoleces into adult worms [12], involving the expression of bile acid receptors and transporters to stimulate the pathways [14]. However, the association between these molecular events and the gradual morphological changes during the strobilar development of *E. granulosus* remain essentially unknown.

In an attempt to finding genes and molecular pathways involved with the gradual phenotypic changes triggered by strobilation stimuli in *E. granulosus*, we reported here the transcriptomic profile for the first 24 hours after protoscolex *in vitro* induction to adult development. We performed a comparative analysis of the transcriptomes from four different time-point samples during protoscolex strobilar induction and provide an overview of the early parasite developmental processes. Our data constitute the basis of future studies aimed at investigating the strobilar development and the host-parasite relationships that could be applied in the improvement of new control strategies for echinococcosis.

## Methods

### Parasite material and *in vitro* cultivation

*E. granulosus* protoscoleces (G1 genotype) were aseptically collected from a naturally infected liver of cattle routinely slaughtered at a commercial abattoir (São Leopoldo, RS, Brazil). The viability of protoscoleces was determined by trypan blue exclusion test and confirmed based on their motility characteristics under light microscopy [17]. Protoscoleces were washed 3 times with PBS, pH 7.4 (Sample 1 – PBS) and genotyped by one-step PCR [18]. PSCs were cultured *in vitro* as previously described [19]. Briefly, protoscoleces were incubated for 15 min with pepsin (2 mg/mL), pH 2.0 (Sample 2 – PEP), washed with PBS and transferred to a biphasic medium contained taurocholate for 12 (Sample 3 – 12 h) or 24 h (Sample 4 – 24 h). Additionally, protoscoleces were maintained in culture to confirm the characteristic morphological changes of strobilar development, as previously described [19].

**RNA extraction**

Total RNA from each parasite sample was extracted using Trizol reagent, according to manufacturer's instructions, followed by treatment with RNase-free DNase I (Sigma) to remove DNA contaminants. The integrity of the extracted RNA was monitored using gel electrophoresis on a 1% agarose gel. RNA concentration was determined using Qubit (Molecular Probes).

**cDNA library construction and sequencing**

For cDNA libraries, 4 μg of total RNA were used as start material. PBS, PEP, 12 h and 24 h sample libraries were constructed, without replicate, using the TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina) according to manufacturer's instructions. Library quality control was performed using the 2100 Bioanalyzer System with the Agilent High Sensitivity DNA Kit (Agilent). The libraries were individually quantified via qPCR using a KAPA Library Quantification Kits for Illumina platforms (KAPA Biosystems). They were pooled together in

equal amounts and sequenced in a MiSeq Sequencing System (Illumina). Paired-end reads (2x75 bp) were obtained using a MiSeq Reagents Kit v3 (150 cycles.)

**Data analysis**

FastQC v0.11.2 [20] was used for data set quality checking. Individual Illumina read files (fastq) were trimmed and filtered using Trimmomatic v0.36 [21]. Paired end Trimmomatic parameters used were: LEADING:10 TRAILING:10 SLIDINGWINDOW:30:20 MINLEN:30. Filtered reads were mapped to *E. granulosus* genome by using TopHat2 v2.1.0 [22]. The genome of *E. granulosus* (PRJEB121, [14]) and annotation (version 2014-05) were retrieved from WormBase ParaSite database [23].

The reads mapped to each transcript were used to calculate normalized transcript abundance and to perform differential gene expression analysis in GFOLD v1.1.4 [24], a software package specifically designed for unreplicated RNA-seq data. Genes with |GFOLD value|>1 or |log2 (fold change)|>2 were considered to be differentially expressed.

Hierarchical cluster analysis and Pearson correlation coefficient was performed using the R Stats Package v3.4.0, corrplot package v0.77 and RStudio v1.0.143. The conserved functional domain structures (SUPERFAMILY, [25]) of the identified differentially expressed (DE) genes were predicted using InterProScan 5.21-60.0 [26]. The eggNOG database v4.5.1 [27] was used to acquire the functional annotation for the DE genes.

**Results**

The paired-end RNA-seq sequencing using Illumina technology was used in order to investigate the transcriptome of protoscoleces induced to strobilar development. The RNA-seq resulted in a total of 30,821,916 reads. The overall raw read mean quality score was high, with 98.4% of bases above Q30. After quality filtering, 30.8 million of paired-end reads (99.5%) were

obtained and 71.7% of the reads were mapped to the *E. granulosus* genome with known gene annotations. Table 1 shows the summary of the sequencing results.

**Table 1. Summary of the *E. granulosus* RNA-seq data.**

| Samples | Q30[a] | Raw reads | Filtered reads | Mapped reads |
|---|---|---|---|---|
| **PBS** | 0.984 | 6,994,764 | 6,965,344 (99.6%) | 4,909,354 (70.5%) |
| **PEP** | 0.983 | 7,348,062 | 7,309,726 (99.5%) | 5,090,978 (69.6%) |
| **12 h** | 0.985 | 8,308,408 | 8,271,920 (99.6%) | 6,103,570 (73.8%) |
| **24 h** | 0.982 | 8,315,180 | 8,274,926 (99.5%) | 6,085,915 (73.5%) |

[a]Q30: Phred Quality Score; probability of incorrect base call: 1 in 1000.

PBS: protoscoleces washed with PBS; PEP: protoscoleces washed with PBS + treatment with pepsin; 12 h: protoscoleces subjected to PBS + PEP + biphasic medium for 12 h; 24 h: protoscoleces subjected to PBS + PEP + biphasic medium for 24 h.

A total of 9376 different genes were present in our dataset. A total of 9019, 9029, 9051 and 9077 genes were found in the PBS, PEP, 12 h and 24 h samples, respectively. Most of the genes (8742 genes) were detected in the four samples analyzed, but 61, 72, 56 and 73 genes were exclusively detected in PBS, PEP, 12 h and 24 h samples, respectively (Figure 1). A complete list of identified genes is provided in Additional file 1.

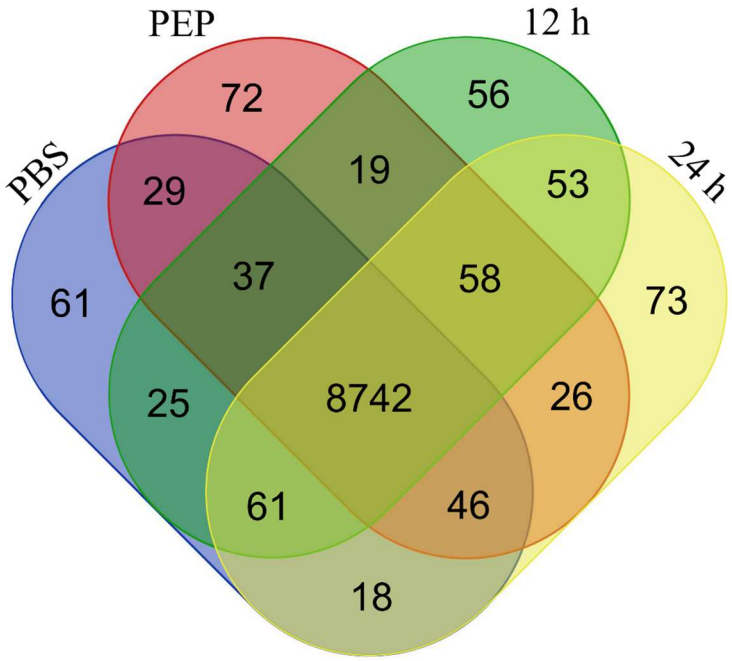


**Figure 1. Venn diagram showing the distribution of detected genes between *E. granulosus* protoscolex samples.** Genes with non-zero RPKM are represented and compared to show the number of genes with overlapping expression at four samples analyzed.

The variation between protoscolex samples was calculated with the Pearson correlation. As shown in Figure 2, PBS and PEP samples show higher correlation coefficients than 12 and 24 h samples, indicating that there were little variation among them and a separation from the other samples.

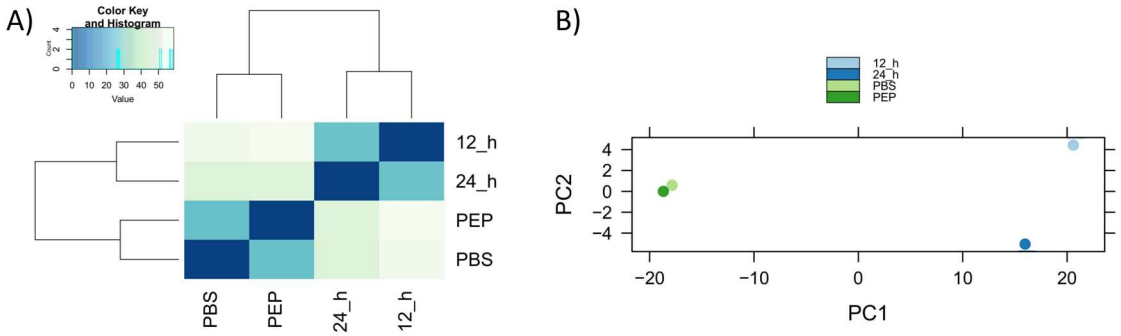


**Figure 2. Correlation analysis of sequenced samples.** (A) Pearson correlation coefficients between samples; (B) Principal Component Analysis.

We performed a pairwise comparison across different samples with GFOLD. A total of 818 genes were differentially expressed between any two samples (Additional file 2). The number of DE genes (up- and downregulated) in each 2x2 sample comparison is shown in Figure 3. A predominance of downregulated genes was detected in all pairwise comparisons. PEP vs 12 h showed the highest amount of DE genes (552 genes, 180 upregulated and 372 downregulated). Some of these data are shown in Table 2. These genes are related to growth and development, immunity, signal transduction, etc, and may be important for strobilation.

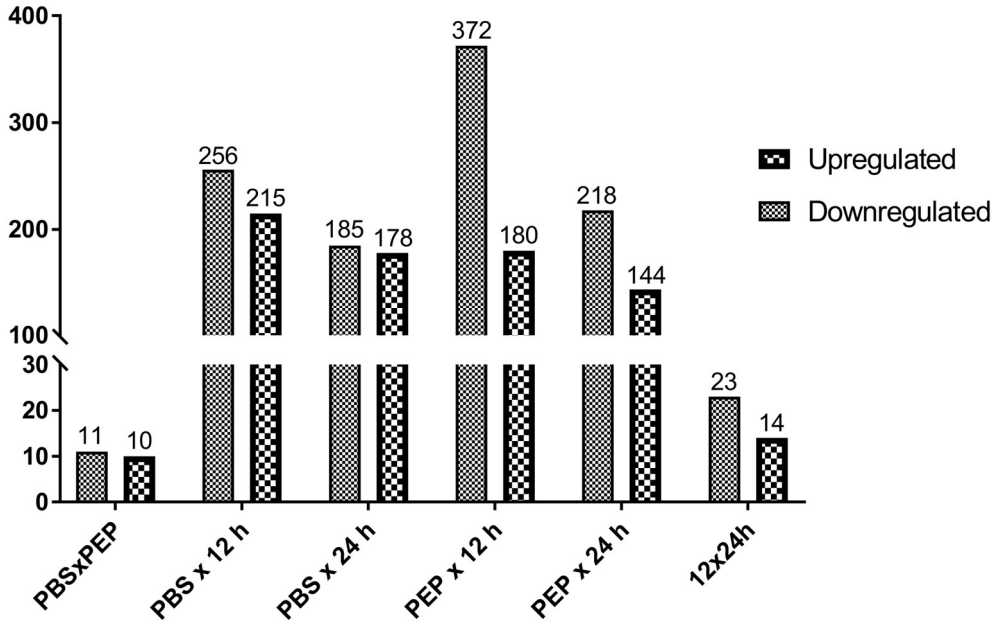


**Figure 3. Differentially expressed genes.** Up- and downregulated genes in each pairwise comparison are shown.

To better understanding of the dynamics of E. granulosus protoscolex gene expression, DE genes were clustered to search for similar patterns of expression between samples (Figure 4). Using the sum of the RPKMs values in the four samples (cutoff > 10), 744 DE genes were categorized into eight different clusters on account of their relative expression pattern (Additional file 2). We computed a relative expression for each gene by dividing its expression at each time point by the sum of gene expression for all time points. In this analysis, clusters 1

and 2, including 446 genes, showed decreasing expression profile patterns throughout the treatment for protoscolex strobilation, while 277 genes in clusters 6, 7 and 8 showed increasing expression profiling patterns.

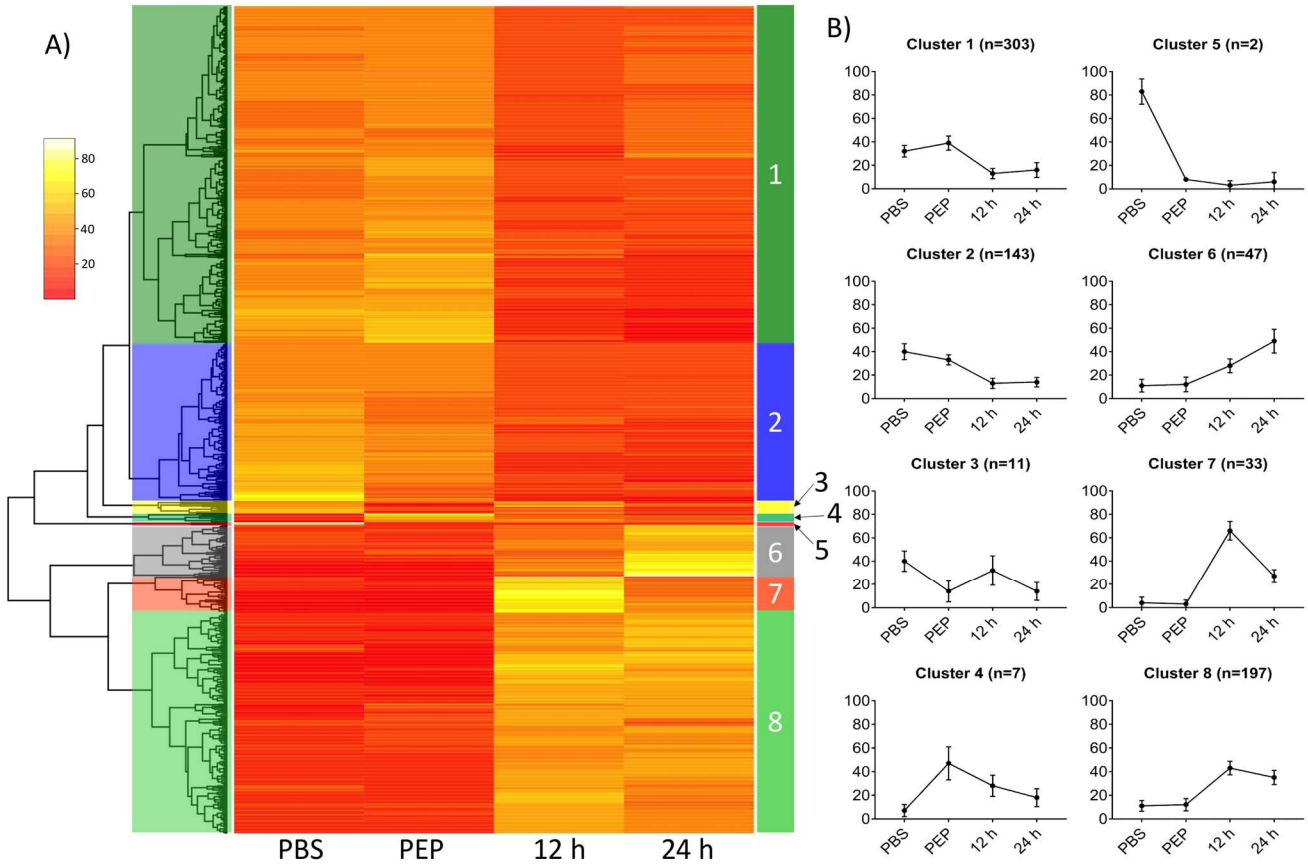


**Figure 4. Heatmap plot of DE genes during *E. granulosus* strobilar development induction.** (A) Relative expression of each gene is represented in the rows and categorized clusters; (B) Average expression (+/− standard deviation) for genes in each cluster reveals similar expression patterns.

**Table 2. Some of the genes that were differentially expressed among the samples analyzed of *E. granulosus*.**

| | | RPKM | | | | Profile | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene_ID** | **Gene_Name** | **ST** | **PEP** | **12h** | **24h** | **PBSx12 h** | **PBSx24 h** | **PEPx12 h** | **PEPx24 h** | **Superfamily[a]** | **EggNOG[b]** |
| EgrG_000107200 | 2 amino 3 ketobutyrate coenzyme A ligase | 201,774 | 203,383 | 1583,37 | 1074,42 | Up | Up | Up | Up | RB | E |
| EgrG_000117000 | Ankyrin | 24,0945 | 17,9373 | 59,5489 | 24,153 | - | - | Up | - | RD | O |
| EgrG_001165500 | Ankyrin | 74,7373 | 67,9019 | 246,727 | 182,952 | Up | Up | Up | Up | RD | E |
| EgrG_000193700 | Annexin | 1095,57 | 1303,16 | 443,858 | 833,11 | Down | - | Down | - | IA | U |
| EgrG_000244000 | Annexin | 872,956 | 973,713 | 303,621 | 434,858 | Down | - | Down | Down | IA | U |
| EgrG_000330300 | Annexin | 953,654 | 973,326 | 467,045 | 427,206 | Down | Down | Down | Down | IA | U |
| EgrG_000124900 | Aquaporin 4 | 195,726 | 234,991 | 28,6044 | 41,8997 | Down | Down | Down | Down | RF | G |
| EgrG_000153200 | Aquaporin 9 AQP 9 small solute channel 1 | 6,07709 | 6,86419 | 325,38 | 156,091 | Up | Up | Up | Up | RF | G |
| EgrG_001190800 | Aquaporin 9 AQP 9 small solute channel 1 | 11,3987 | 8,44232 | 422,027 | 210,038 | Up | Up | Up | Up | RF | G |
| EgrG_000203700 | Choline transporter protein 2 | 72,4286 | 75,4026 | 30,3665 | 33,5379 | Down | - | Down | Down | - | P |
| EgrG_000127200 | Concentrative Na nucleoside cotransporter | 0,472568 | 1,20989 | 287,929 | 113,074 | Up | Up | Up | Up | - | P |
| EgrG_000342900 | Cysticercus cellulosae specific antigenic | 18289,6 | 20966,8 | 117,226 | 204,453 | Down | Down | Down | Down | - | - |
| EgrG_000261100 | Diagnostic antigen gp50 | 9,18014 | 6,89773 | 1,77943 | 2,37523 | Down | - | Down | - | - | - |
| EgrG_000564000 | Diagnostic antigen gp50 | 441,138 | 466,854 | 46,5439 | 81,7405 | Down | Down | Down | Down | - | - |
| EgrG_000520550 | Diagnostic antigen gp50 | 0,446094 | 0 | 8,28655 | 3,982 | Up | Up | Up | Up | - | - |
| EgrG_000304800 | Diagnostic antigen gp50 | 3,21728 | 2,40246 | 161,229 | 133,223 | Up | Up | Up | Up | - | - |
| EgrG_000324200 | Diagnostic antigen gp50 | 104,485 | 115,282 | 568,807 | 422,461 | Up | Up | Up | Up | - | - |
| EgrG_000566700 | Diagnostic antigen gp50 | 145,332 | 149,558 | 497,9 | 427,249 | Up | Up | Up | Up | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EgrG_000071400 | Dynein light chain | 574,633 | 776,583 | 374,921 | 400,581 | - | - | Down | - | N | Z |
| EgrG_000941000 | Dynein light chain | 1522,5 | 2014,49 | 431,851 | 361,5 | Down | Down | Down | Down | N | Z |
| EgrG_000941100 | Dynein light chain | 2298,07 | 3266,25 | 512,348 | 697,2 | Down | Down | Down | Down | N | Z |
| EgrG_000940900 | Dynein light chain | 3620,31 | 4518,12 | 1492,48 | 1444,34 | Down | Down | Down | Down | N | Z |
| EgrG_000347100 | Elongation of very long chain fatty acids | 168,956 | 193,03 | 429,035 | 277,86 | Up | - | - | - | - | I |
| EgrG_000321300 | Elongation of very long chain fatty acids | 145,857 | 168,369 | 379,318 | 343,661 | Up | - | - | - | - | I |
| EgrG_000549800 | Fatty acid binding protein FABP2 | 1195,61 | 1125,07 | 497,465 | 454,409 | Down | Down | Down | Down | RF | I |
| EgrG_000549850 | Fatty acid binding protein FABP2 | 15557,9 | 17680,6 | 7030,25 | 5519,12 | Down | Down | Down | Down | RF | I |
| EgrG_000500800 | Forkhead box protein J3 | 6,74812 | 10,582 | 25,906 | 19,0748 | Up | - | - | - | LA | K |
| EgrG_000159700 | Forkhead box protein K1 | 56,2761 | 55,7012 | 16,4889 | 13,4864 | Down | Down | Down | Down | LA | K |
| EgrG_000179000 | Forkhead box protein O4 | 127,353 | 101,933 | 47,5055 | 44,8686 | Down | Down | Down | Down | LA | K |
| EgrG_000320300 | Forkhead box protein P4 | 84,3874 | 94,8522 | 42,8213 | 33,9381 | - | Down | Down | Down | LA | K |
| EgrG_000166300 | Forkhead box Q:D protein transcription | 57,4808 | 55,836 | 168,579 | 133,053 | Up | - | Up | - | LA | K |
| EgrG_000521000 | Heat shock protein 70 | 10,0821 | 6,12389 | 69,4092 | 45,7634 | Up | Up | Up | Up | RC | O |
| EgrG_000520950 | Heat shock protein 70 | 6,45234 | 6,57028 | 108,38 | 50,6147 | Up | Up | Up | Up | RC | O |
| EgrG_000554100 | Heat shock protein 70 | 37,0527 | 31,2413 | 324,553 | 280,569 | Up | Up | Up | Up | O | O |
| EgrG_000772900 | Homeobox protein ceh 26 | 9,33366 | 9,52123 | 2,16725 | 6,50904 | Down | - | Down | - | LA | K |
| EgrG_000040400 | Krueppel factor 10 | 559,331 | 589,885 | 127,123 | 114,181 | Down | Down | Down | Down | LA | K |
| EgrG_000608700 | Krueppel factor 5 | 317,941 | 424,699 | 35,579 | 28,4103 | Down | Down | Down | Down | LA | K |
| EgrG_000419100 | Kunitz protease inhibitor | 1520,32 | 1792,19 | 769,481 | 648,642 | - | Down | Down | Down | OA | - |
| EgrG_000445600 | Long chain fatty acid coenzyme A ligase 5 | 395,132 | 314,496 | 137,806 | 124,926 | Down | Down | Down | Down | RC | I |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EgrG_000212700 | Major egg antigen p40 | 2923,9 | 3139,22 | 1186,33 | 848,683 | Down | Down | Down | Down | O | O |
| EgrG_000586900 | Oxalate:formate antiporter | 58,5919 | 46,314 | 22,5862 | 26,3801 | Down | - | - | - | P | G |
| EgrG_000586800 | Oxalate:formate antiporter | 9,41304 | 6,93566 | 56,9295 | 24,2628 | Up | - | Up | Up | P | G |
| EgrG_000017400 | Oxalate:formate antiporter | 186,078 | 190,454 | 28,2336 | 36,1629 | Down | Down | Down | Down | P | G |
| EgrG_000661800 | Oxalate:formate antiporter | 248,386 | 231,971 | 33,6004 | 48,2633 | Down | Down | Down | Down | P | G |
| EgrG_000204100 | P53 transcription factor DNA binding | 98,8238 | 81,228 | 39,9723 | 41,4273 | Down | Down | - | - | - | - |
| EgrG_000249100 | Phospholipase d1 | 0,193565 | 0,27876 | 19,9917 | 17,2063 | Up | Up | Up | Up | RC | I |
| EgrG_000204600 | Potassium voltage gated channel subfamily D | 6,35751 | 4,88303 | 0,944766 | 3,31039 | Down | - | - | - | P | P |
| EgrG_000197100 | Proto oncogene serine:threonine protein kinase | 147,414 | 116,318 | 48,2426 | 38,11 | Down | Down | Down | Down | OB | T |
| EgrG_001001900 | Pyruvate kinase | 0,569787 | 2,55289 | 5,50381 | 3,67332 | Up | Up | - | - | C | G |
| EgrG_001127400 | Sodium bile acid cotransporter | 0,767959 | 0,552983 | 5,13557 | 2,85629 | Up | - | Up | - | - | - |
| EgrG_001127500 | Sodium bile acid cotransporter | 13,8243 | 13,0956 | 76,5597 | 56,7643 | Up | Up | Up | Up | - | P |
| EgrG_000381100 | Tapeworm specific antigen B | 18,6673 | 6,72087 | 2,6007 | 1,73574 | Down | Down | - | - | - | - |
| EgrG_000381600 | Tapeworm specific antigen B | 59,6463 | 93,9159 | 252,619 | 231,605 | Up | Up | - | - | - | - |
| EgrG_000381400 | Tapeworm specific antigen B | 705,858 | 551,111 | 25,1401 | 58,1474 | Down | Down | Down | Down | - | - |
| EgrG_000381200 | Tapeworm specific antigen B | 2680,18 | 2204,6 | 171,536 | 310,395 | Down | Down | Down | Down | - | - |
| EgrG_000381700 | Tapeworm specific antigen B | 34,8619 | 42,5989 | 167,195 | 157,953 | Up | Up | Up | Up | - | - |
| EgrG_000381800 | Tapeworm specific antigen B | 140,34 | 217,926 | 2490,87 | 3299,76 | Up | Up | Up | Up | - | - |
| EgrG_000328400 | Tetraspanin | 35,7626 | 42,9192 | 59,7888 | 105,163 | - | Up | - | - | RE | S |
| EgrG_001077400 | Tetraspanin | 131,894 | 137,408 | 308,601 | 578,232 | - | Up | - | Up | RE | S |
| EgrG_000873600 | Universal stress protein | 2596,12 | 3003,99 | 501,033 | 436,72 | Down | Down | Down | Down | F | T |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EgrG_000811100 | Universal stress protein | 13,3471 | 25,0464 | 224,943 | 152,537 | Up | Up | Up | Up | F | T |
| EgrG_001017500 | Zinc finger protein | 50,7582 | 65,6282 | 17,2905 | 17,7426 | Down | Down | Down | Down | LA | K |
| EgrG_000186000 | Zinc finger protein 45 | 13,5889 | 10,3948 | 1,88806 | 2,05454 | Down | Down | Down | Down | LA | K |
| EgrG_000711000 | Zinc finger transcription factor gli2 | 35,5351 | 41,3339 | 14,4482 | 11,1371 | Down | Down | Down | Down | LA | K |
| EgrG_000737300 | Zinc transporter foi | 18,7469 | 24,4141 | 59,7177 | 72,487 | Up | Up | - | Up | - | P |

[a]Superfamily code: C – energy; F - nucleotide metabolism and transport; IA – phospholipid metabolism and transport; LA – DNA-binding; N – cell motility; O – protein modification; OA – proteases; OB - kinases/phosphatases; P – ion metabolism and transport; RB – transferases; RC – other enzymes; RD – protein interaction; RE - immune response; RF – transport.

[b]EggNOG functional categories: E – amino acid transport and metabolism; G – carbohydrate transport and metabolism; I – lipid transport and metabolism; K – transcription; O – post-translational modification, protein turnover, and chaperones; P – inorganic ion transport and metabolism; S – function unknown; T – signal transduction mechanisms; U – intracellular trafficking, secretion, and vesicular transport; Z – cytoskeleton.

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Among the upregulated genes obtained from GFOLD analysis, we identified genes coding for 2-amino-3-ketobutyrate coenzyme A ligase, heat shock protein 70 (16 genes), sodium bile acid cotransporter (2 genes) and tetraspanin (4 genes). In contrast, among the downregulated genes, genes coding for annexin (6 genes), calcium binding protein (2 genes), dynein light chain (10 genes) and fatty acid binding protein FABP2 were identified.

A structural and functional annotation of the DE genes is summarized in Figure 5 (Additional file 2). The most representative domains found in the genes downregulated during early worm development (cluster 1and 2) were related to cell motility, cell adhesion, DNA-binding, translation and DNA replication/repair. Among the genes upregulated during early worm development (cluster 6, 7 and 8), the most representative functions were signal transduction, other enzymes and protein modification. By EggNOG analysis, the most significant functions found in downregulated genes were translation, ribosomal structure and biogenesis (J), intracellular trafficking, secretion, and vesicular transport (U) and cytoskeleton (Z). In the upregulated genes, the most relevant functions were inorganic ion transport and metabolism (P), nucleotide transport and metabolism (F) and amino acid transport and metabolism (E).

Uncharacterized proteins represent 270 of total DE genes, with 68 annotated as expressed conserved proteins, 62 expressed proteins and 140 hypothetical proteins (Additional file 2). Of these, only 10 have conserved domains predicted by SUPERFAMILY and 13 have EggNOG functional categories (three of them present both classifications).

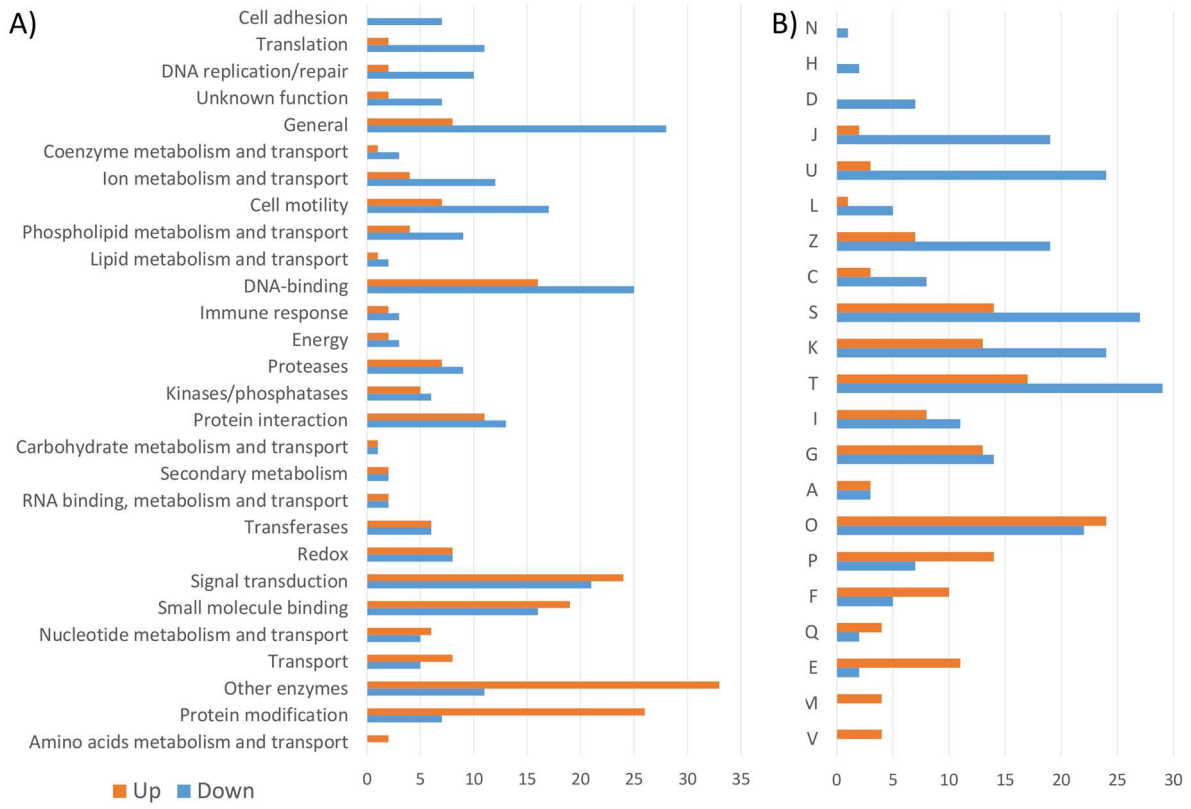


**Figure 5. Comparative structural and functional analysis of upregulated (clusters 6, 7 and 8) and downregulated (clusters 1and 2) DE genes.** (A) SUPERFAMILY predictions of the conserved domains shown by percentage; (B) EggNOG functional categories of DE genes shown by percentage. (A) RNA processing and modification; (C) Energy production and conversion; (D) Cell cycle control, cell division, chromosome partitioning; (E) Amino acid transport and metabolism; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism; (H) Coenzyme transport and metabolism; (I) Lipid transport and metabolism; (J) Translation, ribosomal structure and biogenesis; (K) Transcription; (L) Replication, recombination and repair; (M) Cell wall/membrane/envelope biogenesis; (N) Cell motility; (O) Post-translational modification, protein turnover, and chaperones; (P) Inorganic ion transport and metabolism; (Q) Secondary metabolites biosynthesis, transport, and catabolism; (S) Function unknown; (T) Signal transduction mechanisms;(U) Intracellular trafficking, secretion, and vesicular transport; (V) Defense mechanisms; (Z) Cytoskeleton.

## Discussion

Important morphological and biochemical changes occur throughout the life cycle of parasitic organisms and are probably the result of regulated changes in gene expression in response to

environmental stimuli such as host, temperature and pH changes [28–30]. These regulated responses contribute to the mechanisms by which parasites subvert host immune defenses and cause infection.

Previously, based on the classic works of Smyth and collaborators (summarized in [12]), we have reported an in vitro culture of E. granulosus protoscolex strobilar development based on a biphasic medium containing the bile salt taurocholate [19]. In this work, we cultivate protoscoleces in biphasic medium for 12 or 24 hours. In addition, we used untreated protoscoleces washed with PBS (PBS) and protoscoleces treated with pepsin (PEP) to compare and to search for DE genes involved in the strobilar development.

Based on the Jacob-Monod model, a hypothetical but logical model was proposed to explain how the gene expression regulation can be involved in the Echinococcus development [7,31]. Although both the morphological characteristics of the strobilar development and the genome of E. granulosus are known, the correlation between these two informations and the model previously proposed is still poorly understood. In this work, we provide a transcriptional analysis of E. granulosus protoscoleces in vitro induced to strobilar development in attempt to finding genes involved in this process.

By sample-to-sample correlation analysis, it was possible to observe that the most abundant transcripts share similarities between the different samples analyzed. PBS and PEP samples have a relative high-level expression of several identical gene transcripts. Furthermore, with the subsequent activation of the protoscoleces by pepsin and bile salts, mimicking the developmental transition in the definitive host, a change in the identity of highly expressed genes can be observed.

Among the most expressed genes found in our data, it is important to verify the presence of fatty acid binding protein (FABP) and the antigen B transcripts. Both of them have already been

described among the highly Echinococcus expressed genes [14,32], which corroborates the validity of our data. The importance of these genes lies in the fact that cestodes are unable to synthesize fatty acids and cholesterol de novo (Tsai et al., 2013). They depend essentially on the sequestration and utilization of host lipids by proteins like FABP and the antigen B. In this study, FABP genes are downregulated during E. granulosus adult worm development. Antigen B has both up- and downregulated genes, in agreement with previous works [33,34].

Among the downregulated DE genes, we found several genes coding for dynein light chain, oxalate:formate antiporter and annexins. Dynein is a family of cytoskeletal motor proteins involved in intracellular motility of vesicles and organelles along microtubules and are associated with transforming growth factor (TGF)-β signaling [14,35]. Previous studies showed the expansion of this family in E. granulosus and schistosomes when compared to nematodes [13,14]. Oxalate:formate antiporter is a subfamily of the major facilitator transporter family, responsible for the transport of small solutes [36], but its function is not fully understood in parasites. Annexins, in contrast, are considered to play critical roles in parasite process related to the maintenance of cell integrity and modulation of the host immune responses [37]. Therefore, the decrease in the expression of the annexins may be related to the absence of contact with the host in the in vitro cultures.

On the other hand, we found ankyrin, tetraspanin, heat shock protein 70 (Hsp70) and sodium bile acid cotransporter among upregulated DE genes. Ankyrins are involved in functions such as cell-cycle regulation, transcriptional regulation, cytoskeleton interactions, signal transduction, development and intracellular trafficking [38,39]. In parasites, tetraspanins are involved in the coordination of signal transduction, cell proliferation, adhesion, migration, cell fusion, and host–parasite interactions [40]. In E. granulosus, tetraspanins were mostly present in the tegument and could contribute to the parasite nutrition [41]. The Hsp70 are part of the group of the expanded domain families in E. granulosus and may have important roles in protein

folding and in protecting cells from stress [14]. The expression of Hsp70 may be particularly associated to the stressful conditions of strobilation induction, which involves an increase in protein synthesis [19,42]. In turn, sodium bile acid cotransporter is an integral membrane glycoprotein that, in humans, participate in the enterohepatic circulation of bile acids. Bile acids seem to play a key role in the differentiation of Echinococcus protoscoleces into adult worms, and the expression of bile acid receptors and transporters may be stimulated during strobilar development [12,15].

In our previous work, we identified proteins expressed by E. granulosus protoscoleces upon the induction of strobilar development [19]. These proteins were related to the cytoskeleton, energy metabolism and cellular communication. Specifically, the 2-amino-3-ketobutyrate coenzyme A ligase, classified here as an upregulated DE gene, had an increased expression in the presence of strobilation stimuli.

When we analyzed the molecular function of DE genes, we also found differences between clusters. In cluster 1, which presents a downregulated expression pattern, we observed the presence of more basal functions, such as translation (e.g., Eukaryotic translation initiation factor 5a), DNA replication and cell motility. In contrast, the upregulated DE genes of cluster 8 are related to specialized functions like signal transduction (e.g., tyrosine protein kinase and G protein coupled receptor), enzymes (e.g., hexokinase and phospholipase) and protein modifications (e.g., Hsp70), which might correlate with the increased morphological complexity of the adult tapeworm compared to the metacestode. It is important to note that an expressive number of genes (270 in DE genes; 2976 in total) are not characterized, which difficult more accurate analyses. This is the case, for example, of the hypothetical protein EgrG_000335800, which is one of the most expressed genes in the four conditions analyzed.

**Conclusion**

In this study, we have conducted RNA-Seq analysis of the protoscoleces and early strobilar stages of E. granulosus. More importantly, this work provides information about DE genes in key intermediate stages, providing novel information about E. granulosus strobilar development. In summary, we provide here significant data that can be used to explore basic questions on the biology and evolution of cestodes, including the study of development and the host-parasite relationship.

**Additional file**

Additional file 1: List of identified genes. (XLSX 2000 kb)

Additional file 2: List of differentially expressed genes. (XLSX 98 kb)

**Abbreviations**

DE: differentially expressed; FABP: fatty acid binding protein; HSP: heat shock protein; RPKM: reads per kilobase million.

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**Availability of data and materials**

All sequence data (raw Illumina reads) are available on the NCBI Sequence Read Archive (SRA) database under the accession ID SRP131874.

**Author's contributions**

JAD, KMM and AZ conceived and designed the experiments. JAD and ALG performed the experiments. JAD, KMM and AZ analyzed the data. JAD, ALG, ATRV and AZ contributed to the writing of the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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# 5. Discussão geral, conclusões e perspectivas

Um dos maiores desafios na vida de um parasito é passar de um hospedeiro para outro. No caso do *E. granulosus*, os estágios de desenvolvimento se alternam entre hospedeiros intermediário e definitivo, onde o parasito será exposto a diferentes microambientes. A regulação da expressão gênica provavelmente permite a sobrevivência a esses diferentes estímulos, através de alterações metabólicas e fenotípicas que são observadas ao logo do ciclo de vida.

Muito antes da era genômica, diversos estudos acerca da biologia parasitária e da relação parasito-hospedeiro foram publicados. Através deles, foram conhecidas as condições necessárias para estimular o desenvolvimento dos estágios larval e adulto *in vitro* (sumarizados em SMYTH, 1990); a existência do rostelo e a importância do contato com o intestino, ou um substrato semelhante a este, para o desenvolvimento em verme adulto (SMYTH; HOWKINS; BARTON, 1966; MORSETH, 1967; SMYTH; GEMMELL; SMYTH, 1970); a plasticidade no desenvolvimento dos protoescólices de *Echinococcus*, que os permite desenvolver-se em verme adulto ou em cisto hidático secundário, além da influência de fatores ambientais no controle desses processos (SMYTH; HOWKINS; BARTON, 1966; SMYTH; MILLER, 1967; SMYTH; MILLER; HOWKINS, 1967); e os sais biliares como um fator determinante para a especificidade e o estabelecimento do parasito no hospedeiro definitivo (SMYTH, 1968). Embora já tenha sido proposto um modelo hipotético para explicar como os genes regulam o desenvolvimento em *Echinococcus* (SMYTH, 1969), a correlação dessas informações com os dados do genoma e transcritomas e a identificação de genes/proteínas com papel-chave no processo de estrobilização ainda se encontra pendente.

Os protoescólices são um dos principais alvos de pesquisas em *Echinococcus* uma vez que são encontradas diversas dificuldades práticas e éticas na realização de estudos *in vivo* utilizando o hospedeiro definitivo (THOMPSON et al., 2006). Porém, o cultivo de *Echinococcus* ainda não está totalmente adaptado a rotina laboratorial, sendo possível manter apenas parte de seu ciclo de vida em laboratório. Dessa forma, a obtenção dos parasitos é dependente de infecções naturais e, como demonstrado anteriormente pelo nosso grupo, a grande maioria dos cistos hidáticos coletados de bovinos é infértil, ou seja, não apresenta protoescólices (BALBINOTTI et al., 2012).

Outra dificuldade encontrada na utilização de *E. granulosus* como modelo de estudo está no fato de que as publicações são, na sua maioria, da década de 1960, ou seja, antes da revisão taxonômica do gênero. A incerteza da espécie utilizada e a quantidade de protocolos diferentes existentes fizeram com que um dos nossos primeiros objetivos fosse a revisão e a definição de um modelo de cultivo que fosse capaz de fornecer as condições fisiológicas necessárias para o desenvolvimento estrobilar. Além disso, foram necessárias modificações adicionais para tornar possível a incorporação de azido-homoalanina, como a remoção do extrato de levedura, a diminuição na concentração de soro fetal bovino e utilização de meio de cultivo sem metionina. Apesar de tais modificações, esse protocolo de cultivo foi eficaz em induzir os protoescólices ao desenvolvimento estrobilar (DEBARBA et al., 2015), uma vez que os protoescólices que foram mantidos em cultivo apresentaram alterações fenotípicas compatíveis com o desenvolvimento estrobilar, como a diminuição do número de corpúsculos calcários e o surgimento dos canais excretores e da bexiga excretória posterior.

Após definir o protocolo de cultivo e indução, utilizamos duas diferentes abordagens técnicas com o objetivo de identificar genes e mecanismos moleculares que possam estar envolvidos nas alterações fenotípicas que são observadas após os protoescólices de *E. granulosus* receberem estímulos para a estrobilização. No primeiro trabalho, proteínas recém-sintetizadas foram marcadas com azidohomoalanina, aminoácido análogo da metionina, purificadas através de cromatografia de afinidade e identificas por espectrometria de massas. Este trabalho resultou na identificação de 365 proteínas, das quais 75 foram diferencialmente expressas na comparação entre a presença e ausência de estímulos de estrobilização e 51 foram expressas de forma exclusiva em uma condição ou outra. Já na segunda abordagem, utilizamos a técnica de RNA-seq para realizar uma análise do transcritoma de protoescólices que foram tratados com pepsina e cultivados por até 24 horas em meio bifásico contendo o sal biliar taurocolato. Aqui, 818 genes foram considerados diferencialmente expressos entre as quatro condições analisadas.

Como já citado anteriormente, a dependência de infecções naturais para a obtenção de protoescólices constitui uma dificuldade adicional para a obtenção de réplicas biológicas. Para experimentos de RNA-seq, é recomendado que se tenha um mínimo de três réplicas biológicas para que a análise de expressão diferencial possua poder estatístico (CONESA et al., 2016). Apesar disso, a presença de experimentos sem réplicas ainda é bastante frequente. Em 2011, no banco de dados do Gene Expression Omnibus, por volta de 70% das amostras de RNA-seq humanas não possuíam réplicas biológicas, além de, também naquele ano, essas terem sido

depositadas em maior número em comparação com a presença de réplicas (BARRETT et al., 2011; FENG et al., 2012). Para contornar esse viés, a análise utilizou o software GFOLD, cujo algoritmo é especialmente desenhado para experimentos que não apresentam tais réplicas (FENG et al., 2012). Como resultado de tal análise, é obtido um ranqueamento dos genes diferencialmente expressos com base na distribuição posterior do logaritmo do *fold change*. Embora seja uma ferramenta relativamente nova, o GFOLD já foi utilizado e citado por diversos pesquisadores, além de ter gerado bons resultados na comparação com outras ferramentas de análise de dados de RNA-seq (DOU et al., 2015; JIA et al., 2015; KHANG; LAU, 2015). Adicionalmente, o padrão de expressão de genes como a FABP e as subunidades de antígenos B correlacionam-se com estudos anteriores, o que representa uma garantia indireta da validade dos dados.

Com relação aos resultados obtidos, de forma geral, protoescólices sem tratamento ou sem estímulos para a estrobilização apresentam um predomínio de genes e proteínas relacionados a funções celulares básicas, como a regulação de processos biológicos e a organização de componentes celulares. Em contraste, a presença de estímulos para a estrobilização é acompanhada de alterações na expressão gênica do parasito, tornando-o apto para sobreviver e se desenvolver no microambiente do hospedeiro definitivo.

Uma característica visível em diferentes parasitos obrigatórios é a redução de vias metabólicas, uma vez que o parasito adquire grande parte de seus nutrientes a partir do hospedeiro (TSAI et al., 2013). Dessa forma, alterações em genes/proteínas envolvidos com processos metabólicos são um importante indicativo da transição de estágios, fornecendo indícios de como o parasito obtém energia durante esse período. Dentre as proteínas identificadas em nosso trabalho, encontramos a fosfoenolpiruvato carboxiquinase (PEPCK), que é descrita em diferentes cestódeos por atuar na produção de oxaloacetato a partir de fosfoenolpiruvato (SMYTH; MCMANUS, 1989). Por outro lado, em vertebrados superiores, a PEPCK atua principalmente no processo de gliconeogênese. Considerando, portanto, a importância da enzima na obtenção de energia, a inibição de PEPCK poderia diminuir a utilização de glicose pelos parasitos, constituindo um potencial alvo anti-helmíntico (DAS et al., 2015; KUMAR DUTTA et al., 2017). Encontramos, também, a proteína citrato sintase. O citrato é considerado um regulador clássico da glicólise através de seu efeito inibitório sobre a fosfofrutoquinase (MCMANUS; SMYTH, 1978; SMYTH; MCMANUS, 1989). Embora esses mecanismos não sejam totalmente esclarecidos, acredita-se que o mesmo ocorra em *E. granulosus*. Por fim, encontramos ainda a proteína 2-amino-3-quetobutirato coenzima A ligase,

cujo gene que a codifica foi considerado diferencialmente expresso em nossa análise de RNA-seq. De modo geral, tais alterações no metabolismo estão de acordo com estudos anteriores, os quais verificaram que estágios mais avançados do desenvolvimento de vermes apresentam uma prevalência do metabolismo mitocondrial, cujo rendimento energético é maior (KITA; HIRAWAKE; TAKAMIYA, 1997; CONSTANTINE et al., 1998; MAZET et al., 2013; MILLERIOUX et al., 2013).

Grande parte do nosso conhecimento acerca de processos bioquímicos, moleculares e celulares advém de estudos envolvendo um pequeno número de organismos modelo. Sendo assim, quando consideramos um organismo como o *E. granulosus*, cujo desenvolvimento apresenta características únicas, é acrescido um grau adicional de dificuldade para a realização da correta correlação entre genes e funções. Por isso, é importante mencionar que algumas enzimas, especialmente as que estão envolvidas no metabolismo de carboidratos, são conhecidas por realizar diversas atividades *moonlighting* (HUBERTS; VAN DER KLEI, 2010; LORENZATTO et al., 2012; JEFFERY, 2015), ou seja, podem apresentar múltiplas funções bioquímicas ou biofísicas fisiologicamente relevantes, que são distintas da função tida como clássica. Entre as proteínas *moonlighting* já descritas e que foram identificadas em nosso trabalho temos a fosfoenolpiruvato carboxiquinase – envolvida na manutenção do equilíbrio redox e na indução da imunidade mediada por células T (ASAHI et al., 2000); citrato sintase – atividade de chaperona, além de envolvimento no crescimento e virulência (BUCHNER; GRALLERT; JAKOB, 1998); e frutose 1,6 bisfosfato aldolase – atuação na adesão celular e como antioxidante (LORENZATTO et al., 2012).

Com a publicação do genoma de *E. granulosus* e de outras três espécies de cestódeos (TSAI et al., 2013), verificou-se que aproximadamente 20% dos genes eram exclusivos. Entre os genes exclusivos desses organismos estão famílias de antígenos altamente expressos como o antígeno B, proteínas ancoradas por glicosilfosfatidilinositol (GPI) GP50 e o antígeno EG95, que é um alvo para vacina (LARRIEU et al., 2015). Dos cinco genes que codificam o antígeno EG95, apenas um foi identificado (EgrG_002001600), ainda que com baixo nível de expressão. Por outro lado, identificamos 9 dos 10 genes preditos para GP50, sendo que quatro deles (EgrG_000304800, EgrG_000324200, EgrG_000564000 e EgrG_000566700) apresentam um valor de RPKM maior que 100 em pelo menos uma das amostras analisadas. Além disso, seis deles foram considerados diferencialmente expressos durante a estrobilização, o que remete ao observado em *T. solium*, organismo no qual GP50 apresenta um comportamento estágio-específico em *Cysticercus cellulosae* (HANCOCK et al., 2004). Com relação ao antígeno B, 6

dos 7 genes preditos foram identificados e considerados diferencialmente expressos. Adicionalmente, o padrão de expressão observado para esses genes é o mesmo que foi verificado anteriormente pelo nosso grupo, através de PCR quantitativa (ESPÍNOLA; FERREIRA; ZAHA, 2014). Curiosamente, nenhum desses antígenos foi identificado como uma proteína recém-sintetizada. Uma possível explicação para isso, além de serem antígenos secretados ou de membrana, está no fato de que esses antígenos possuem um papel central no processo de evasão da resposta imune do hospedeiro, a qual não é requerida por se tratar de um cultivo *in vitro*, com condições controladas.

Entre os estímulos que desencadeiam o processo de estrobilização, a bile teria um papel fundamental por estimular a evaginação do escólex de cestódeos levando à diferenciação, bem como atuar de forma sinérgica com enzimas proteolíticas para digerir membranas do cisto (SMYTH; HASLEWOOD, 1963). Além disso, a concentração de ácidos biliares poderia, juntamente com outros fatores, estar relacionada com a determinação da especificidade do hospedeiro para *E. granulosus* (SMYTH, 1968). *E. granulosus* pode expressar receptores e transportadores de ácidos biliares para estimular vias importantes para seu desenvolvimento, incluindo o *farnesoid X receptor* (FXR) e o receptor de vitamina D (VDR) (ZHENG et al., 2013). Ao ser ingerido pelo hospedeiro, os protoescólices entram em contato com ácidos biliares, que são importados pelo transportador de ácido biliar e podem se ligar e ativar o *FXR–retinoid X receptor* (RXR), o que leva à ativação transcricional do receptor nuclear. Do mesmo modo, os ácidos biliares podem estimular a dimerização e a ativação de VDR-RXR. Considerando que utilizamos o sal biliar taurocolato como um estímulo para a estrobilização, é animador encontrar dentre as proteínas recém-sintetizadas a proteína *SNW domain-containing protein*, que, em humanos, codifica um coativator de transcrição que pode interagir com o VDR-RXR (FOLK; PŮTA; SKRUŽNÝ, 2004); e o gene *sodium bile acid cotransporter*, que codifica uma glicoproteína de membrana e cuja função está relacionada ao transporte de sais biliares (HAGENBUCH; MEIER, 1994).

Por fim, o significativo número de genes e proteínas não caracterizados constitui um importante resultado e uma interessante perspectiva. Foram 270 genes diferencialmente expressos encontrados com essa condição, além de 36 proteínas recém-sintetizadas. Quatro proteínas expressas conservadas (EgrG_000087900, EgrG_000696700, EgrG_000701500, EgrG_0010619000) e uma proteína hipotética (EgrG_002052200) apresentam tanto o gene quanto a proteína identificados nesse trabalho como expressos em resposta ao desenvolvimento estrobilar. Cinco proteínas hipotéticas (EgrG_000600100, EgrG_000658900,

EgrG_000785000, EgrG_000990300, EgrG_002052200) tem em nosso trabalho, pela primeira vez, evidência experimental de tradução. A caracterização desse grupo de proteínas em um genoma leva à descoberta de novas estruturas e funções, além de ajudar a desvendar vias e cascatas proteicas, complementando o conhecimento básico sobre um organismo (NIMROD et al., 2008; IJAQ et al., 2015). Considerando as particularidades do desenvolvimento de *E. granulosus*, assim como outros organismos cujas patologias são negligenciadas, este pode ser um importante ponto de partida para a busca de marcadores moleculares e alvos farmacológicos.

As análises anteriores ficaram concentradas na comparação entre protoescólices e verme adulto, que, embora tenha sua relevância, não explora a questão básica da transição entre esses dois estágios. Ao utilizarmos dois níveis diferentes de regulação da expressão gênica, este trabalho acrescenta uma grande quantidade de dados com possíveis indícios de genes-chave que se correlacionam com as mudanças fenotípicas esperadas. Fica como uma perspectiva, então, a realização de mais estudos para comprovar a participação desses alvos no processo de estrobilização, além de identificar suas funções específicas. Adicionalmente, dada a dificuldade de trabalhar com parasitos, os alvos moleculares, mecanismos relacionados e técnicas utilizadas no estudo de protoescólices de *E. granulosus* poderiam ser extrapolados para outros cestódeos e servir como base para o estudo de métodos de diagnóstico e tratamento. Ainda, dada a escassez de estudos mais aprofundados utilizando parasitos como modelo de estudo, é importante considerar os diversos genes e proteínas que não estão caracterizados, sem falar que a investigação de novas funções gênicas pode ser importante não só no contexto da estrobilização, mas explicar pontos importantes da adaptação ao parasitismo.

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*Curriculum vitae* **- resumido**

**DEBARBA, JA**

## 1. DADOS PESSOAIS

**Nome:** João Antonio Debarba
**Local e data de nascimento:** Sananduva, RS, Brasil, 07/12/1987
**Endereço profissional:** Irmandade da Santa Casa de Misericórdia de Porto Alegre
Rua Prof. Annes Dias, 295
Centro Histórico - Porto Alegre, RS, Brasil
90020-090
**Telefone profissional:** +55 51 3214 8080
**E-mail:** joaoatd@gmail.com

## 2. FORMAÇÃO:

**2013** Doutorado em Biologia Celular e Molecular.
UFRGS, Porto Alegre, Brasil
Orientador: Arnaldo Zaha
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

**2011 - 2013** Mestrado em Biologia Celular e Molecular.
UFRGS, Porto Alegre, Brasil
Título: Marcação e detecção de proteínas sintetizadas durante o desenvolvimento estrobilar de *Echinococcus granulosus in vitro*
Orientador: Arnaldo Zaha
Bolsista do: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

**2006 - 2009** Graduação em Biomedicina.
UFCSPA, Porto Alegre, Brasil
Título: Construção de um bacmídio recombinante a ser empregado em um sistema baculovírus/células Sf9 para produção de proteínas farmacologicamente ativas derivadas de veneno.
Orientador: Ana Beatriz Gorini da Veiga
Bolsista do(a): Universidade Federal de Ciências da Saúde de Porto Alegre

## 3. ESTÁGIOS:

**Irmandade da Santa Casa de Misericórdia de Porto Alegre - ISCMPA**

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**Vínculo institucional**

**2009 - 2009** Vínculo: Estagiário curricular, Carga horária: 30

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**Atividades**

**07/2009 - 10/2009** *Estágio: Estágio curricular no Banco de Sangue, totalizando 500 horas.*

**01/2009 - 05/2009** *Estágio: Estágio curricular no Laboratório Central de Análises Clínicas, totalizando 500 horas.*

**Universidade Federal do Rio Grande do Sul - UFRGS**

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**Vínculo institucional**

**2007 - 2007** Vínculo: Estagiário, Carga horária: 20

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**Atividades**

**05/2007 - 12/2007** Estágio, Instituto de Ciências Básicas e da Saúde, Departamento de Bioquímica

*Estudo ontogenético dos efeitos da homocisteína sobre a fosforilação de proteínas do citoesqueleto em córtex e hipocampo de ratos.*

**Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA**

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**Vínculo institucional**

**2006 - 2009** Vínculo: Estagiário curricular, Carga horária: 20

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**Atividades**

**01/2009 - 11/2009** *Estágio no Laboratório de Biologia Molecular*

**09/2006 - 12/2006** *Estágio voluntário no Laboratório de Patologia da Pós-graduação da FFFCMPA*

## 4. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA ANTERIOR

**Irmandade da Santa Casa de Misericórdia de Porto Alegre - ISCMPA**

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**Vínculo institucional**

**2017 -** Vínculo: Celetista, Enquadramento funcional: Biomédico, Carga horária: 36

**HemoCord - Banco de Sangue de Cordão Umbilical - HEMOCORD**

**Vínculo institucional**

**2010 - 2010** Vínculo: Celetista, Enquadramento funcional: Biomédico, Carga horária: 44

**Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA**

**Vínculo institucional**

**2006 - 2009** Vínculo: Monitor, Carga horária: 20

**Atividades**

**03/2009 - 06/2009** Graduação, Biomedicina
*Monitoria de Biologia Molecular*

**03/2007 - 06/2007** Graduação, Biomedicina
*Monitoria de Química*

## 5. ARTIGOS COMPLETOS PUBLICADOS EM PERIÓDICOS

1. **DEBARBA, J. A.;** MONTEIRO, K. M.; MOURA, H. ; BARR, J. R. ; FERREIRA, H. B. ; ZAHA, A. . Identification of Newly Synthesized Proteins by *Echinococcus granulosus* Protoscoleces upon Induction of Strobilation. **PLoS Neglected Tropical Diseases (Online),** v. 9, p. e0004085, 2015.

## 6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

1. **DEBARBA, J. A.**; MONTEIRO, K. M.; FERREIRA, H. B.; ZAHA, A. Análise proteômica de protoescólices de *Echinococcus granulosus* induzidos à estrobilização. In: **XXIV Congresso Brasileiro de Parasitologia e XXIII Congreso Latinoamericano de Parasitología – FLAP**, Salvador. 2015.

2. **DEBARBA, J. A.**; MONTEIRO, K. M.; FERREIRA, H. B.; ZAHA, A. Specific Labeling of Newly Synthesized Proteins in *Echinococcus granulosus*. In: **XLIII Encontro Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular**, Foz do Iguaçu. 2014.

3. **DEBARBA, J. A.**; MONTEIRO, K. M.; FERREIRA, H. B.; ZAHA, A. Marcação, detecção e visualização de proteínas sintetizadas durante o desenvolvimento estrobilar de *Echinococcus granulosus*. In: **XXIII Congresso Brasileiro de Parasitologia e III Encontro de Parasitologia do Mercosul**, Florianópolis. 2013.

4. **DEBARBA, J. A.**, CARMO, A. C. V., CIBULSKI, S. P., SUAZO, C. A. T., MENDONCA, R. Z., VEIGA, A. B. G. Construção de bacmídios recombinantes para a expressão de proteínas de interesse farmacológico do veneno de *Lonomia obliqua* em células Sf9. In: **XXi Salão de Iniciação Científica da UFRGS**, 2009.

5. HOLZ, C. L., **DEBARBA, J. A.**, CIBULSKI, S. P., TEIXEIRA, T. F., CAIXETA, S. P. M. B., BATISTA, H. B. C. R., CAMPOS, F. S., ROEHE, L. R., OLIVEIRA, M. T., SILVA, J. R., DEZEN, D., VARELA, A. P. M., KUNERT FILHO, H. C., FRANCO, A. C., ROEHE, P. M. Soroprevalência de anticorpos neutralizantes para a infecção pelo herpesvírus bovino no Estado do Rio Grande do Sul. In: **XVIII Congresso Mundial de Epidemiologia - VII Congresso Brasileiro de Epidemiologia**. , 2008.

6. VANZIN, C. S., LOUREIRO, S. O., HEIMFARTH, L., ZAMONER, A., PIEROZAN, P., **DEBARBA, J. A.**, LACERDA, B. A., SANTOS, N. G., VIDAL, L. F., WYSE, A., PUREUR, R. P. A homocisteína causa hiperfosforilação dos filamentos intermediários de hipocampo de ratos In: **XIX Salão de Iniciação Científica da UFRGS,** 2007, Porto Alegre.

7. SANTOS, N. G., LOUREIRO, S. O., HEIMFARTH, L., PIEROZAN, P., **DEBARBA, J. A.**, LACERDA, B. A., VANZIN, C. S., VIDAL, L. F., PUREUR, R. P., ZAMONER, A. Ações não genômicas da vitamina D3 sobre a fosforilação da vimentina via mecanismos dependentes de cálcio em testículos de ratos imaturos. In: **XIX Salão de Iniciação Científica da UFRGS,** 2007, Porto Alegre.

8. LACERDA, B. A., ZAMONER, A., LOUREIRO, S. O., HEIMFARTH, L., PIEROZAN, P., **DEBARBA, J. A.**, VANZIN, C. S., VIDAL, L. F., SANTOS, N. G., PUREUR, R. P. Citoesqueleto como alvo das ações não genômicas da tiroxina em córtex cerebral de ratos jovens In: **XIX Salão de Iniciação Científica da UFRGS**, 2007, Porto Alegre.

9. BERNARDI, R. B., **DEBARBA, J. A.**, SAMBRANO, G. E., MATTES, P. E., LOPES, F. M., MULLER, C.B., BACK, F. P., CRESTANI, T. A. Estudo etológico de ratos epiléticos tratados ou não com carbamazepina e testados no campo aberto, labirinto em cruz eelvado e nado forçado. In: **XXII Reunião Anual da Federação de Sociedades de Biologia Experimental, FeSBE**, 2007.

10. **DEBARBA, J. A.**, LOUREIRO, S. O., HEIMFARTH, L., ZAMONER, A., PIEROZAN, P., VANZIN, C. S., VIDAL, L. F., LACERDA, B. A., SANTOS, N. G., WYSE, A., PUREUR, R. P. Estudo ontogenético dos efeitos da homocisteína sobre a fosforilação de proteínas do citoesqueleto em córtex e hipocampo de ratos In: **II Semana Acadêmica da FFFCMPA**, 2007.