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

Análise comparativa do transcritoma de dois estágios no desenvolvimento do parasito cestódeo *Mesocestoides corti*

Tese de Doutorado

Tatiana Noel Basika Cabrera

Porto Alegre, novembro de 2016

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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 Biologia Celular e Molecular

Tatiana Noel Basika Cabrera

Orientador: Dr. Henrique Bunselmeyer Ferreira

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“...com o último adeus recitou-nos este verso do Mantuano, particularmente próprio para viajantes incertos do caminho

Et quacumque viam dederit fortuna sequamur”

Julio Verne
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LISTA DE ABREVIATURAS

AGO – argonauta (*argonaute*)

BiNGO – *Biological Networks Gene Ontology*

BLAST – *Basic Local Alignment Search Tool*

BMP – proteína morfogenética do osso (*bone morphogenetic protein*)

Ca^{2+} – cálcio

CDS – sequência codificadora

CO_2 – dióxido de carbono

DNA – ácido desoxirribonucleico

cDNA – DNA complementário

DNAse – desoxirribonuclease

EGF – fator de crescimento epidérmico (*epithelial growth factor*)

FPKM – fragmentos por quilobase de exon por milhões de fragmentos mapeados (*fragments per kilobase of exon per million fragments mapped*)

GAPDH – gliceraldeído-3-fosfato desidrogenase (*glyceraldehyde 3-phosphate dehydrogenase*)

GO – *Gene Ontology*

GST – glutationa-S-transferase (*glutathione-S-transferase*)

Hsp70 – proteina de choque térmico de 70 kDa (*heat shock protein 70 kDa*)

IGF – fator de crescimento semelhante à insulina (*insulin-like growth factor*)

MAP – proteina cinase ativada por mitógeno (*mitogen activated protein kinase*)

Mb – milhões de pares de bases

NCBI – *National Center for Biotechnology Information*

NFκB – fator nuclear kappa B (*factor nuclear kappa B*)

bp – pares de bases

PBS – solução salina tamponada com fosfato

RISC – complexo de silenciamento induzido por RNA (*RNA-induced silencing complex*)

RNA – ácido ribonucleico

mRNA – RNA mensageiro

miRNA – microRNA

rRNA – RNA ribossômico

RNAse – ribonuclease

RNA-seq – sequenciamento de RNA

RTKs – receptores tirosina cinases (*receptor tyrosine kinases*)

ST – verme estrobilado

TGF- β – fator de transformação do crescimento β (*transforming growth factor β*)

TT – tetratirídeo

Wnt – *wingless*

USP – proteínas de estresse universal (universal stress proteins)

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RESUMO

O desenvolvimento em cestódeos abrange mudanças fisiológicas e morfológicas complexas. Com o intuito de ganhar conhecimento nos mecanismos moleculares que regulam este processo, foi realizada uma análise exaustiva da expressão de microRNAs e genes em dois estágios do desenvolvimento (tetratirídeo e verme estrobilado) do cestódeo *Mesocestoides corti*. Utilizando uma abordagem de sequenciamento de alto rendimento, foi identificada evidencia transcricional para 42 loci de microRNAs, assim como microRNAs diferencialmente expressos ou estágios específicos. Além disso, foi demonstrado que a uridilação é um mecanismo de modificação pós-transcricional diferencial presente nos microRNAs de *M. corti*. O conjunto completo de microRNAs de *M. corti* identificado representa 33 famílias únicas, e confirma a notável perda de famílias de microRNAs conservadas dentro do grupo de platelmintos parasitos. O transcritôma de *M. corti* foi obtido utilizando uma abordagem baseada em sequenciamento de RNA. Foram evidenciados 19,053 transcritos, incluindo isoformas e genes previamente não anotados. Foram identificados um total de 66 transcritos específicos de tetratirídeo e 136 específicos de verme estrobilado, assim como genes diferencialmente expressos (342 e 559 respectivamente), sugerindo regulação de caraterísticas específicas de cada estágio. A analise de expressão diferencial e enriquecimento de termos GO refletem os processos biológicos associados a cada estágio, sendo o tetratirídeo capaz de reproduzir-se assexuadamente e migrar através dos tecidos do hospedeiro intermediário, e o verme estrobilado de maturar e reproduzir-se sexuadamente no hospedeiro definitivo. Globalmente, os resultados apresentados fornecem uma plataforma para estudos de novos mecanismos moleculares da regulação gênica pós-transcricional baseados em microRNAs em cestódeos, necessários para a elucidação de aspectos do desenvolvimento da complexa biologia destes parasitos. Além disso, este trabalho significativamente contribui ao conhecimento do perfil de expressão gênica de *M. corti* aumentando o número de transcritos sequenciados identificados através da anotação funcional de vários genes.

ABSTRACT

Cestode development involves complex morphological and physiological changes. To gain knowledge in the molecular pathways that regulate this process, a comprehensive analysis of microRNAs and genes expressed in two developmental stages (tetrathyridium and strobilated worm) of the model cestode *Mesocestoides corti* was performed. Using a high-throughput sequencing approach, transcriptional evidence of 42 microRNA loci, as well as differentially expressed and stage-specific miRNAs between these developmental stages were found. Moreover, it was shown that uridylation is a differential mechanism of post-transcriptional modification of *M. corti* microRNAs. The whole set of *M. corti* microRNAs represent 33 unique miRNA families, and confirm the remarkable loss of conserved miRNA families within platyhelminth parasites. The *M. corti* transcriptome was obtained through a RNA-seq-based approach. Transcriptional evidence for 19,053 transcripts was found, including isoforms and previously not annotated genes. A total of 66 tetrathyridium and 136 strobilated-specific transcripts were found, as well as differentially expressed genes (342 and 559, respectively), suggesting regulation of stage-specific features. Differential expression and GO term enrichment analysis reflects the biological processes associated with each stage, the tetrathyridium stage being able to reproduce asexually and actively migrate through the intermediate host tissue; and the strobilated worm that undergoes sexual maduration and reproduction in the definitive host. Overall, the presented results provide a valuable platform to studies aiming to identify and characterize novel miRNA-based molecular mechanisms of post-transcriptional gene regulation in cestodes, necessary for the elucidation of developmental aspects of the complex biology of these parasites. In addition, this work significantly contributes to the knowledge of the gene expression profile of *M. corti* by increasing the number of sequenced transcripts identified and through functional annotation of several genes.

1. INTRODUÇÃO

O filo Platyhelminthes é constituído por organismos bilatérios, protostômios, triploblásticos e achatados dorsoventralmente. Os platelmintos são os primeiros animais na escala evolutiva a apresentarem segmentação corporal (Figura 1). Eles são organismos acelomados, que possuem um sistema nervoso simples e centralizado, intestino cego e carecem de órgãos circulatórios, esqueleto e sistema respiratório (MARTÍN-DURÁN; EGGER, 2012). O filo inclui tanto organismos de vida livre (grupo Turbellaria) como parasitos (grupo Neodermata). Dentro do grupo Neodermata, todos os organismos são parasitos obrigatórios pertencentes às classes Monogenea (ectoparasitos), Trematoda e Cestoda (endoparasitos) (OLSON; TKACH, 2005). Há mais de 1.000 espécies de platelmintos conhecidas, a maioria parasita, e praticamente todas as espécies de vertebrados são suscetíveis à infecção por pelo menos uma delas (OLSON et al., 2012).

A regeneração e a reprodução assexuada, baseada num sistema de células totipotentes (neoblastos), estão presentes nos diversos grupos de platelmintos. Enquanto alguns grupos mais basais como os policlados (Turbellaria) retêm o padrão ancestral de clivagem em espiral, a maioria dos platelmintos diverge significativamente deste padrão e exibe estratégias únicas para especificar o padrão do plano corporal adulto. Inicialmente considerados como os bilatérios mais basais, atualmente os platelmintos foram posicionados robustamente no clado Spiralia (Lophotrocozoa). Por tanto, o estudo da embriologia dos platelmintos é importante para uma melhor compreensão da diversificação dos bilatérios e de seus mecanismos de desenvolvimento (MARTÍN-DURÁN; EGGER, 2012).

1.1. Classe Cestoda

Os cestódeos (platelmintos da Classe Cestoda) são os platelmintos parasitas mais especializados e todos eles possuem pelo menos um (e frequentemente mais de um) hospedeiro intermediário, além do hospedeiro definitivo (que aloja a forma adulta). As cestodíases estão entre as helmintíases mais prevalentes em todo o mundo, com destaque para a hidatidose (ou equinococose), causada pela

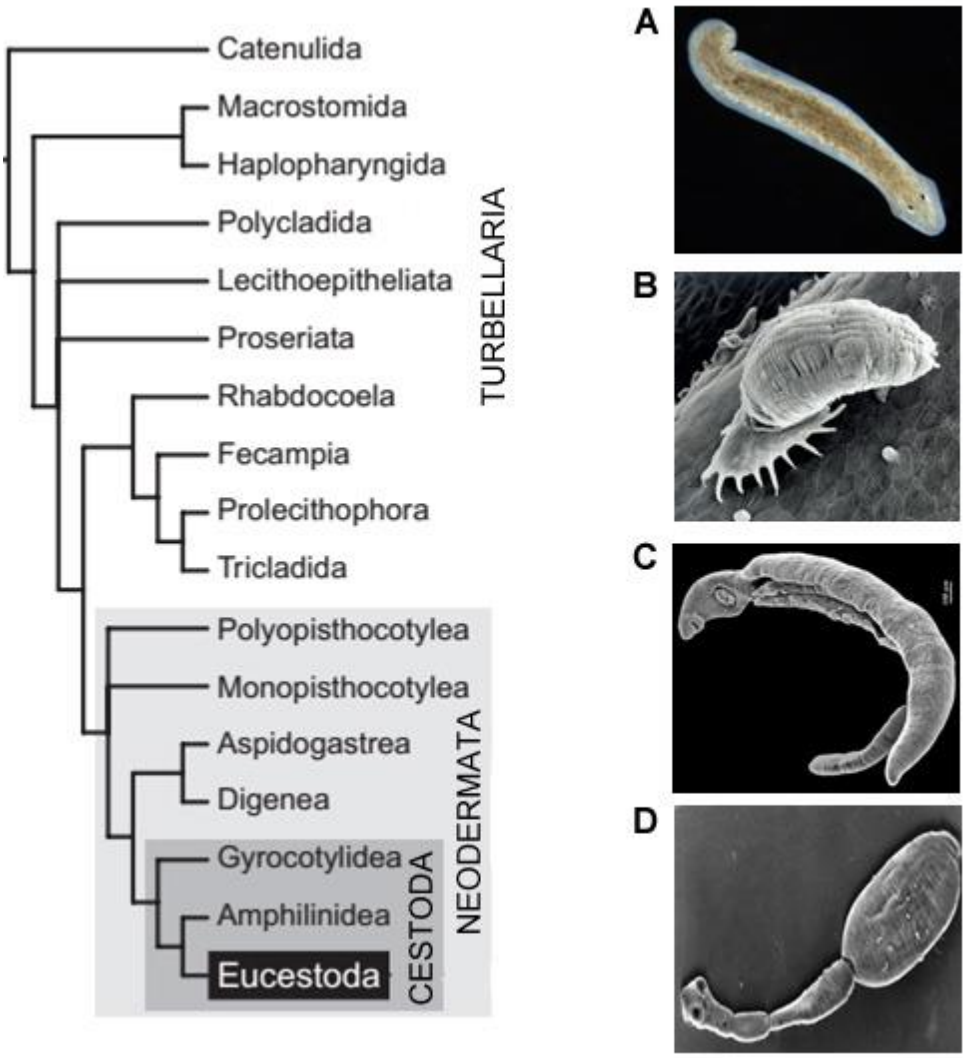

Figura 1 – Filogenia do filo Platyhelminthes – Representação esquemática da classificação evolutiva do filo Platyhelminthes, organismos achatados dorsoventralmente. O filo é composto por organismos de vida livre (Turbellaria) e parasitos (Neodermata). Dentro do grupo Neodermata todos os organismos são parasitos obrigatórios pertencentes às classes Monogenea (ectoparasitos), Trematoda e Cestoda (endoparasitos). Na esquerda se apresenta a filogenia proposta por LITTLEWOOD (2006). Na direita se apresentam exemplos dos organismos pertencentes ao filo: (A) *Schmitdea mediterranea*, turbelária de vida livre; (B) *Gyrodactylus salaris*, monogênea ectoparasito; (C) *Schistosoma mansoni*, trematódeo endoparasito; (D) *Echinococcus granulosus*, cestódeo endoparasito. (Modificado OLSON et al., 2012.)

forma larval (metacestódeo) de espécies do gênero *Echinococcus*, e a cisticercose, causada pelo metacestódeo de *Taenia solium* (TORGERSON;

MACPHERSON, 2011; OTERO-ABAD; TORGERSON, 2013; LIGHTOWLERS, 2013). Em nível mundial, existem aproximadamente 40 milhões de pessoas infectadas por espécies do gênero *Echinococcus*, e 400.000 pessoas infectadas com *T. solium* apenas na América Latina (HEMPHILL, 2010).

Os cestódeos se diferenciam dos outros grupos de platelmintos por carecerem de boca, faringe, esófago/intestino, em todos os estágios do seu desenvolvimento (MAULE; MARKS, 2006). A forma de obtenção do alimento é por absorção através da neoderme, que se encontra modificada com microtríquias que melhoram a absorção. Os cestódeos possuem uma larva (hexacanta) com três pares de ganchos que auxiliam sua fixação no primeiro hospedeiro intermediário, quando ingerida por ele. O desenvolvimento de uma segunda forma larval (metacestódeo) ocorre no hospedeiro intermediário e gera a forma adulta no hospedeiro definitivo. A maioria dos cestódeos adultos possui uma região de fixação, o escólex, que geralmente possui ganchos e ventosas. A partir da base do escólex, se desenvolve uma serie de proglótides, dando ao animal uma aparência de segmentado. As proglótides mais distais do escólex são as mais velhas, mais desenvolvidas e frequentemente contêm os ovos. Este padrão corporal não é uma segmentação verdadeira, mas uma repetição seriada de proglótides durante a estrobilização, sendo a estrobilização a formação do estróbilo ou segmentos. O processo de estrobilização compreende dois eventos, considerados por alguns autores como independentes (LITTLEWOOD, 2008): a proglotização, que corresponde à repetição serial dos órgãos reprodutivos, e a segmentação, que consiste na subdivisão externa das proglótides. As proglótides da maioria dos cestódeos são hermafroditas, com um ou mais conjuntos de órgãos reprodutivos masculinos e femininos. A o incremento da diversidade taxonômica dos cestódeos sugere uma tendência evolutiva de aumento da fecundidade e da complexidade das estruturas de fixação no escólex, ambas presumivelmente associadas à evolução dos ciclos de vida dos cestódeos envolvendo adaptações a hospedeiros de diferentes táxons de animais (HOBERG et al., 1997; OLSON et al., 2001).

1.2. Segmentação e proglotização na classe Cestoda

Assim como os trematódeos da subclasse Digenea, a classe Cestoda desenvolveu, ao longo de sua evolução, uma estratégia própria para atingir uma enorme capacidade reprodutiva. Enquanto os digêneos aumentam sua progênie através de várias gerações assexuadas (rédias e esporocistos), os cestódeos têm aumento na sua fecundidade através de uma repetição seriada de seus órgãos reprodutivos, a proglotização (OLSON et al., 2001). O fenômeno de estrobilização em diferentes ordens de cestódeos é de interesse particular para o estudo da evolução e diversidade de processos de segmentação em metazoários. A ordem Caryophyllidea, considerada a mais primitiva em uma análise filogenética sistemática do grupo (OLSON et al., 2001), é a única que não apresenta proglotização nem segmentação, ao passo que a ordem Spathebothriidea, que compreende organismos ainda muito pouco conhecidos, possui proglotização sem segmentação externa (Figura 2). A proglotização pode aumentar dramaticamente a fecundidade, enquanto que a segmentação, quando seguida da apólise das proglótides maduras, pode permitir que o desenvolvimento e a fertilização ocorram em nichos distintos daqueles ocupados pelo verme parental (OLSON; TKACH, 2005).

1.3. Mecanismos moleculares do desenvolvimento em cestódeos

O estudo da biologia do desenvolvimento de espécies parasitas e da interação entre parasitos e hospedeiros é fundamental para o entendimento do parasitismo. Muitas das transições entre estágios no ciclo de vida de um parasito são iniciadas e controladas por sinais derivados do hospedeiro e a elucidação destas vias de sinalização pode fornecer informações relevantes para o desenvolvimento de drogas anti-helmínticas, vacinas e ferramentas de diagnóstico (BREHM et al., 2006). No caso dos cestódeos, as transformações das formas larvais são eventos-chave no desenvolvimento e são influenciados pelo hospedeiro, que também influencia o tropismo que cada parasito possui pelo órgão de alojamento, seja no fígado (*Echinococcus multilocularis*), fígado ou pulmão (*Echinococcus granulosus*), como no músculo ou cérebro (*T. solium*).

O entendimento dos mecanismos moleculares que sustentam a plasticidade no desenvolvimento é importante para o conhecimento da natureza do parasitismo em cestódeos.

1.3.1. Genômica de parasitos

Inicialmente, as abordagens de biologia molecular em cestódeos se concentravam na identificação de genes codificadores de proteínas antigênicas (ITO, 2002), ou tinham o objetivo de aprofundar-se nos conhecimentos sobre filogenia e epidemiologia (LE et al., 2002). Em anos mais recentes, contudo, tem-se observado enormes avanços no sequenciamento de genomas, na caracterização de proteomas e em outros estudos em grande escala ('ômicos') de helmintos parasitos, em particular de cestódeos (THOMPSON; LYMBERY, 2013).

Tsai et al. (2013) caracterizaram os genomas de quatro tênias: *E. multilocularis*, *E. granulosus*, *Hymenolepis microstoma* e *T. solium*. Posteriormente, Zheng e colaboradores (2013), sequenciaram o genoma de *E. granulosus* reportando achados similares aos de Tsai et al. (2013). Os cestódeos estudados possuem genomas com tamanhos entre 115 Mb e 141 Mb, aproximadamente três vezes menores que os de trematódeos e aproximadamente nove vezes menores que os de platelmintos de vida livre. Os tamanhos relativamente menores se devem principalmente à presença regiões intergênicas menores, ao menor tamanho de íntrons, ao menor conteúdo de sequências repetidas e de elementos gênicos móveis em genomas de cestódeos (ZHENG, H. et al., 2013; TSAI et al., 2013).

Em análises comparativas de genomas de cestódeos e também de genomas de trematódeos têm sido evidenciados eventos de perda ou aquisição de genes associados a adaptações ao parasitismo. Os genomas de cestódeos carecem de vias completas de síntese de pirimidinas, de purinas e da maioria dos aminoácidos. Além disso, cestódeos também não são capazes de sintetizar ácidos graxos e colesterol *de novo* (ZHENG, H. et al., 2013; TSAI et al., 2013). Essas moléculas precisam ser captadas do hospedeiro pelo parasito e, consequentemente, as famílias gênicas associadas a essa captação se encontram expandidas, como por exemplo, famílias de genes codificadores de

proteínas de ligação a ácidos graxos (FABPs) e a família de genes codificadores de subunidades do antígeno B, proteínas que estariam envolvidas na captação e transporte de lipídeos (TSAI et al., 2013).

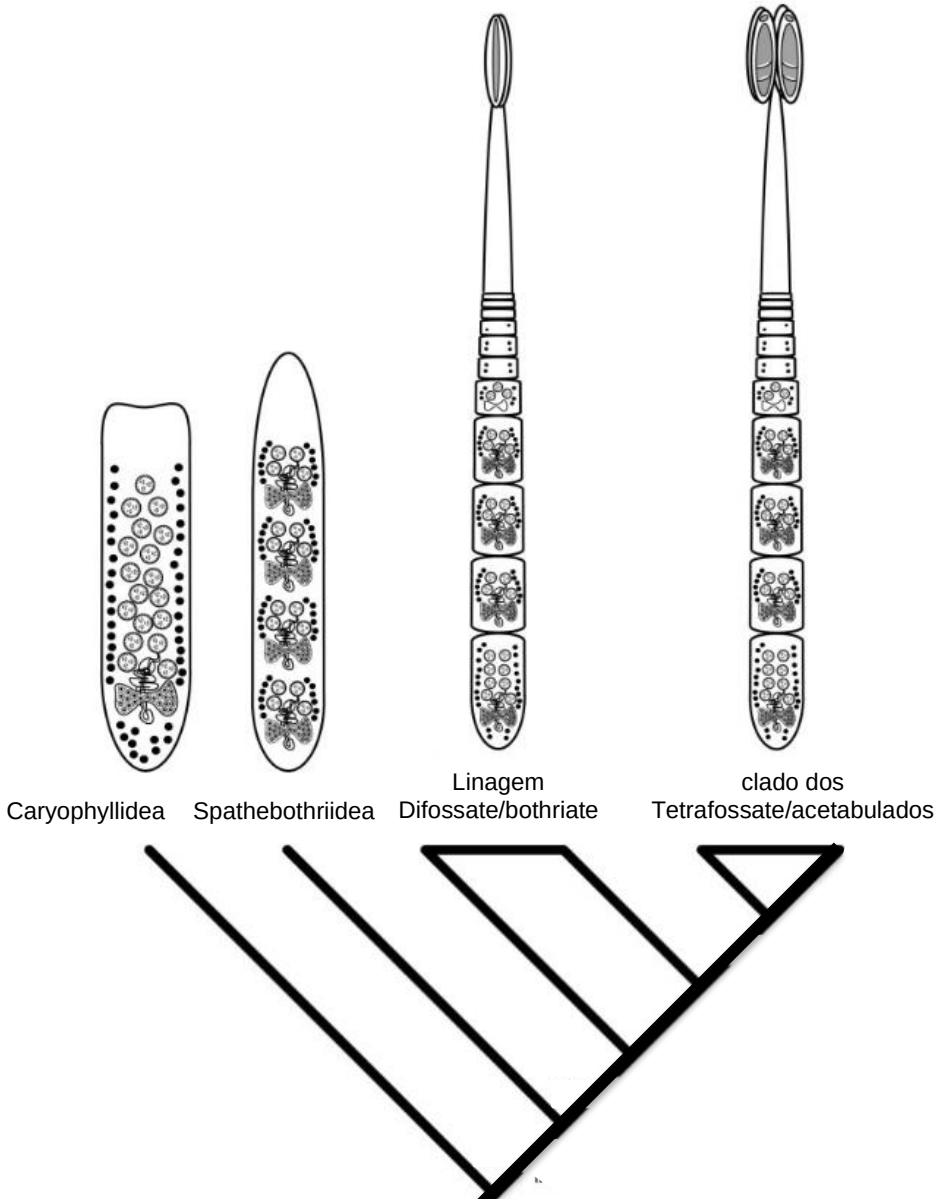

Figura 2 – Representação do padrão de evolução da estrobilização em cestódeos. A ordem Caryophyllidea, mais primitiva de acordo com Olson (2001), é a única que não apresenta proglotização nem segmentação. A ordem Spathebothriidea possui proglotização sem segmentação externa. A segmentação e proglotização é uma condição presente desde o grupo dos Difossate. (Modificado de OLSON et al., 2001).

Outra expansão notável é a observada na família de genes da chaperona molecular Hsp70. Em espécies do gênero *Echinococcus* foram identificadas 22 cópias, majoritariamente localizadas em regiões subteloméricas (ZHENG, H. et al., 2013; TSAI et al., 2013). Este clado expandido carece das caraterísticas clássicas das Hsp70 citosólicas, por tanto se desconhece se a função destes produtos génicos está associada à interação parasito-hospedeiro, como as Hsp70 clássicas, ou se sua localização próxima aos telômeros é importante para sua função ou expressão.

Outro aspecto de relevância para a interação parasito-hospedeiro e para a biologia do desenvolvimento é a presença de sistemas de sinalização evolutivamente conservados, como os componentes das cascatas de transdução de sinais do fator de crescimento epidérmico (EGF), do fator de transformação do crescimento β (TGF-β) e da insulina (BREHM et al., 2006; BREHM, 2010; OLSON et al., 2012).

Também se tem observado reduções nos complementos gênicos de cestódeos implicados na simetria dos planos corporais de metazoários. Os cestódeos apresentam redução do conjunto de genes Hox, perda de vários genes ParaHox (KOZIOL et al., 2009; TSAI et al., 2013) e um conjunto reduzido de ligantes associados a *wingless* (Wnt; RIDDIFORD; OLSON, 2011). Portanto, os cestódeos sofreram significativas reduções dos genes pertencentes a vias de desenvolvimento relacionadas à complexidade do plano corporal durante a evolução do parasitismo.

1.3.2. Vias de sinalização conservadas no desenvolvimento de cestódeos.

Várias das proteínas componentes das vias de sinalização do desenvolvimento têm sido principalmente estudadas em *E. multilocularis* e tem sido iniciado estudos em outros membros da classe Cestoda.

Os peptídeos do tipo EGF são um grupo de mitógenos envolvidos em mecanismos de comunicação célula-célula, que regulam a proliferação e o desenvolvimento de metazoários (GEER, VAN DER et al., 1994; SPILIOTIS et al., 2003). Atuam pela união a receptores tirosina cinases (RTKs) localizados na superfície das células alvo, iniciando uma cascata de sinalização a jusante do receptor, que envolve a via das proteínas cinase ativadas por mitogeno (MAP).

Elementos cruciais destas cascatas incluem GTPases da família Ras e serina/treonina cinases como Raf, MEK e ERK (GEER, VAN DER et al., 1994). Em *E. multilocularis* foram identificados homólogos aos EGF de mamíferos (BREHM et al., 2000) e o seu correspondente receptor (Egfd e EmER, respetivamente), expressos nos estágios de metacestódeo e no protoescólex (SPILIOTIS et al., 2003). Ainda não foi caraterizada a interação entre ambas as moléculas, mas Egfd se mostrou sobre-expressa sob condições que promovem o crescimento e diferenciação do parasito, o que poderia indicar que a sinalização via EGF é um dos mecanismos de desenvolvimento chave nas larvas de cestódeos (BREHM et al., 2006). Adicionalmente, foram caraterizadas em nível molecular e bioquímico GTPases da família Ras, EmRas e EmRal, o ortólogo da cinase Raf (EmRaf), e dos ortólogos de MEK e ERK em *E. multilocularis* e uma proteína tipo ERK em *T. crassiceps* (SPILIOTIS et al., 2006; ESCOBEDO et al., 2010; GELMEDIN et al., 2010).

Os peptídeos relacionados à insulina (como o IGF) também constituem fatores de crescimento hormonal que regulam importantes processos como metabolismo, controle do crescimento e envelhecimento (GEER, VAN DER et al., 1994). As vias de sinalização associadas a estes fatores incluem tirosina cinases da família dos receptores de insulina que ativam várias vias a jusante, incluindo as MAP cinases. O primeiro receptor de IGF descrito em platelmintos foi o EmIR, descrito em *E. multilocularis* (KONRAD et al., 2003). Esta proteína possui atividade tirosina cinase típica desta família de receptores, e similar estrutura de domínios, com exceção de um trecho de 172 aminoácidos no domínio intracelular, não identificado em homólogos. Se especula que o EmIR poderia ter um papel na interação hospedeiro-parasito, interagindo com a insulina do hospedeiro. Em relação a isso, a insulina humana possui um efeito no crescimento de *Mesocestoides vogae* (CANCLINI; ESTEVES, 2009) e estimula a reprodução *in vitro* dos cisticercos de *Taenia crassiceps* (ESCOBEDO et al., 2009). Adicionalmente, foram identificados possíveis peptídeos tipo insulina e os principais componentes da via de sinalização destes receptores por meio de uma busca *in silico* nos genomas de 4 cestódeos, *E. granulosus*, *E. multilocularis*, *T. solium* e *H. microstoma* (WANG et al., 2014).

As citocinas do tipo TGF-β regulam o destino celular por meio da regulação da proliferação, a diferenciação e a apoptose em metazoários (SHI;

MASSAGUE, 2003). A via de sinalização associada a estas citocinas envolve receptores de superfície que consistem em duas serina/treonina cinases transmembrana, chamadas receptores tipo I e tipo II. Após a ativação, este complexo tipo I/II recruta, fosforila e ativa transdutores de sinal intracelulares da família Smad que se translocam ao núcleo para regular a expressão gênica. Em *E. multilocularis* tem sido identificadas três proteínas da família Smad, EmSmadA, EmSmadB, e EmSmadC expressas no metacestódeo e protoescólices (ZAVALA-GÓNGORA et al., 2003, 2008). Adicionalmente foram identificadas duas em *E. granulosus*, a homologa à EmSmadC, EgSmadC, e uma EgSmaD (ZHANG et al., 2014). Estas proteínas apresentam similaridades funcionais e estruturais com as Smad de mamífero. Também foi caraterizada uma serina/treonina cinase de superfície, EmTR1, e também um ligante derivado da família dos TGF-β e BMPs, EmBMP1, que possivelmente seria o ligante cognato de EmTR1 (BREHM, 2010).

1.3.3. Genes reguladores da simetria axial

Os genes Hox codificam fatores de transcrição que estabelecem a polaridade anteroposterior, a diferenciação regional e a elaboração axial por meio da regulação da expressão génica espacial e temporal (LEE et al., 2006), enquanto os genes Wnt codificam ligantes envolvidos em comunicação célula-célula e existe a hipótese de que constituem o sistema ancestral de padrão de desenvolvimento (simetria) de metazoários, que evoluiu para funcionar em conjunto com os genes Hox durante a embriogênese (RYAN; BAXEVANIS, 2007). Em conjunto, essas famílias gênicas e são os reguladores conhecidos mais importantes da simetria axial dos metazoários (RYAN; BAXEVANIS, 2007).

Os genes Hox são notáveis não somente pela sua universalidade na regulação da simetria axial em animais, mas também pela arquitetura “colinear”, pela qual a ordem na que eles estão dispostos no genoma correspondem com os domínios de expressão espacial, anterior, central e posterior (KMITA; DUBOULE, 2003). Os platelmintos possuem um gene anterior (*Hox/Lab*), três genes centrais (*Hox3, Hox4/Dfd, Lox4/Abd-A*) e os posteriores caraterísticos de lofotrocozoários (Post-1/2; NOGI; WATANABE, 2001; OLSON, 2008). De um total de 96 famílias de genes Hox que se supõe que existiram na origem de

Bilateria, 24 não se encontram presentes em trematódeos e cestódeos, e adicionalmente 10 foram perdidos em cestódeos, constituindo o complemento de genes Hox mais reduzido dos animais bilatérios. Entre as perdas específicas de cestódeos se encontram os genes envolvidos no desenvolvimento neuronal (*mnx*, *pax3/7*, *gbx*, *hbn* e *rax*). Isto surpreende já que os cestódeos possuem um sistema nervoso bem desenvolvido, embora possuam sistema sensorial e cefalização reduzidos (TSAI et al., 2013).

Os genes Wnt codificam glicoproteínas de secreção, com 300 a 450 aminoácidos, e caraterizadas pela presença de 23-25 resíduos de cisteína e homologia com os genes *wingless* (*wg*) de *Drosophila* e o gene murino *Intl* (CADIGAN; NUSSE, 1997). A sinalização mediada por Wnt atua em conjunto com os genes Hox no estabelecimento da simetria do eixo anteroposterior (AP) durante a embriogênese (RYAN; BAXEVANIS, 2007), Desta forma, é possível que representem o mecanismo ancestral de simetria em animais, por terem surgido evolutivamente antes do que os genes Hox (PIRES-DASILVA et al., 2003; RICHARDS; DEGNAN, 2009). A partir de dados genômicos, se conhece que em platelmintos parasitos a diversidade de genes Wnt está reduzida, com uma perda de 7 subclasses (RIDDIFORD; OLSON, 2011). Já em cestódeos, recentemente foi demostrado em *E. multilocularis* e *H. microstoma* a expressão de proteínas Wnt na região posterior da larva (hm-wnt1, hm-wnt11b, e o receptor *frizzled* hm-frzd4) e que a formação do escólex é precedida pela expressão localizada de inibidores de Wnt (KOZIOL et al., 2016).

Outro conjunto de genes envolvidos no padrão de simetria axial são os genes ParaHox. Alguns ortólogos dos genes ParaHox tem sido descritos em platelmintos de vida livre (SALÓ et al., 2001), porém cestódeos carecem dos genes ParaHox (*gsx*, *pdx* e *cdx*) ancestralmente envolvidos na especificação do tubo digestivo (TSAI et al., 2013). Interessantemente, o gene posterior ParaHox atua a jusante da via de sinalização Wnt na segmentação de *Drosophila* e camundongo (MARTIN; KIMELMAN, 2009), portanto, se a via Wnt está envolvida na segmentação de cestódeos, a falta de genes ParaHox demostra que o mecanismos é modificado do mecanismo de segmentação canônico de bilatérios (OLSON et al., 2012).

1.4. microRNAs

Os pequenos RNA reguladores pertencentes a classe dos microRNAs (miRNAs) foram originalmente descobertos e descritos como reguladores da expressão gênica de metazoários (BEREZIKOV, 2011). Eles estão envolvidos em diversos processos biológicos, incluindo o desenvolvimento (IVEY; SRIVASTAVA, 2015). Os miRNAs são RNAs endógenos de ~ 23 nt de extensão e executam sua função reguladora por meio de pareamento com mRNAs-alvo para repressão pós-transcricional (BARTEL et al., 2004).

1.4.1. Biogênese e mecanismos de ação de miRNAs

Na via canônica de biogênese (Figura 3), os miRNAs são transcritos pela RNA polimerase II gerando os miRNAs primários (pri-miRNAs) que são processados no núcleo por uma ribonuclease III (Drosha), gerando um pequeno grampo característico, chamado de pre-miRNA ou precursor (~ 70 nt). Com o auxílio do receptor de transporte nuclear (exportina-5) que atua na presença de moléculas com estrutura de grampo e extremidades salientes na extremidade 3', o pre-miRNA é transportado ao citoplasma. No citoplasma, Dicer, outra proteína RNAse III, cliva o precursor produzindo um pequeno duplex de ~ 21-22 nt. No duplex, a sequência madura é a funcional, enquanto a fita complementar é chamada de miRNA *star* (miRNA*; HAMMOND, 2015). Este duplex miRNA-miRNA* é carregado no complexo de silenciamento induzido por RNA (RISC), onde a proteína de união ao RNA é um membro da família de proteínas Argonauta (AGO; FALLER; GUO, 2008). Após, a fita *star* é degradada, porém, em alguns casos, ambas as fitas podem ser incorporadas ao RISC (RO et al., 2007). A sequência madura guia as proteínas AGO a sequências complementares de mRNA para reprimir sua expressão. A sequência que compreende os nucleotídeos da extremidade 5' do miRNA maduro corresponde a sequência *seed*, chave para a interação miRNA-mRNA (BARTEL, 2009; FRIEDMAN et al., 2009). Os miRNA maduros são ativamente regulados por edição, o que pode influenciar sua estabilidade e função. Um dos mecanismos de edição de miRNAs envolve a adição de nucleotídeos (como uridina) na extremidade 3', mecanismos conhecido como *tailing*, que possivelmente induz a degradação da molécula (AMERES et al., 2010).

Diversas evidências experimentais indicam que os miRNAS estão funcionalmente envolvidos com múltiplos processos biológicos, como por exemplo, diferenciação de células-tronco, mudanças de fase, sinalização celular, resposta a estresses bióticos e abióticos e câncer (BARTEL, 2009; HAMMOND, 2015). Os miRNAs são também importantes em processos de desenvolvimento,

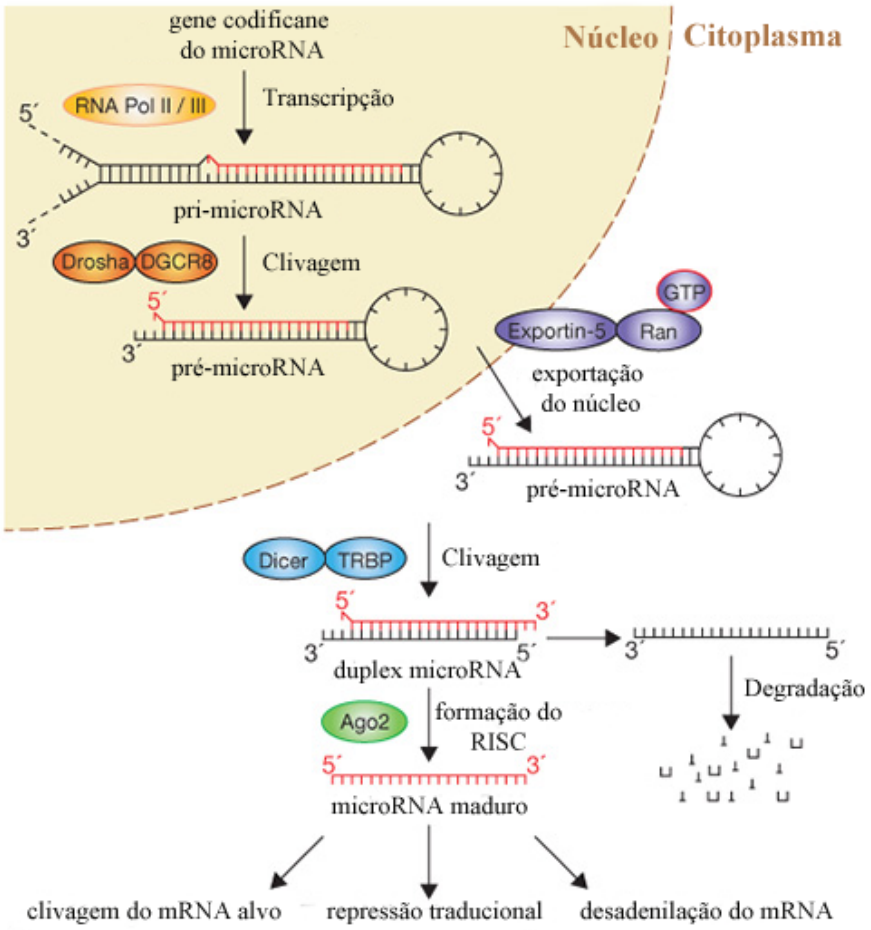

Figura 3 – Via canônica de biogênese dos microRNAs. Os miRNAs são transcritos pela RNA polimerase II gerando os miRNAs primários (pri-microRNAs) que são processados no núcleo por uma ribonuclease III (Drosha), gerando um pequeno grampo característico, chamado de pré-microRNA. São exportados do núcleo pela exportina-5. No citoplasma, Dicer, outra proteína RNAse III, cliva o precursor produzindo um pequeno duplex de ~21-22nt. Este duplex miRNA-miRNA* é carregado no complexo de silenciamento induzido por RNA (RISC). Após, a fita *star* é degradada e sequência madura guia as proteínas AGO a sequências complementares de mRNA para reprimir sua expressão. (Modificado de WINTER et al., 2009).

como evidenciado pelos padrões de expressão espaciais e temporais definidos para muitos deles, e pelos defeitos de divisão e diferenciação em células germinativas na morfogênese embrionária observados em mutantes de perda de

função em enzimas envolvidas na biogênese de miRNAs (IVEY; SRIVASTAVA, 2015). Vários genes de miRNAs já foram também propostos como reguladores de pluripotência das células-tronco embrionárias, da diferenciação celular durante a hematopoiese, da expressão gênica assimétrica em neurônios e do estabelecimento de simetria mediada por genes Hox (FINNEGAN; PASQUINELLI, 2013).

1.4.2. miRNAs na classe Cestoda

Com a exceção de alguns organismos unicelulares, a maquinaria do RISC encontra-se conservada entre os eucariotos (CERUTTI; CASAS-MOLLANO, 2008). Dentro do filo Platyhelminthes, a maquinaria do RISC tem sido identificada tanto em organismos de vida livre (turbelários), como em espécies parasitas (cestódeos e trematódeos), indicando que a regulação da expressão gênica por meio dos miRNAs está presente neste filo (SOUZA GOMES, DE et al., 2013; ZHENG, Y. et al., 2013; ZHENG, 2013).

Entre os platelmintos, miRNAs foram inicialmente identificados em planárias (PALAKODETI et al., 2006; FRIEDLÄNDER et al., 2009) e em trematódeos do gênero *Schistosoma* (CAI et al., 2011). Em cestódeos, esses estudos tem sido limitados principalmente a espécies de importância econômica como *Echinococcus spp.* e *Taenia spp*. O repertorio de miRNAs de *E. granulosus*, *E. multilocularis* e de *Echinococcus canadensis* tem sido recentemente atualizado (BAI et al., 2014; CUCHER et al., 2015; MACCHIAROLI et al., 2015; KAMENETZKY et al., 2016), porém os dados disponíveis para outros cestódeos, como *Taenia saginata* e *Taenia multiceps*, são fragmentados ou ausentes (AI et al., 2012; WU et al., 2013).

1.5. *Mesocestoides corti*

Mesocestoides corti é um verme parasito pertencente à classe Cestoda, filo Platyhelminthes. A forma adulta se encontra no intestino de vertebrados carnívoros. As larvas (tetratirídeos) se encontram na cavidade peritoneal de répteis, aves e mamíferos (WITENBERG, 1934). O tetratirídeo, que possui uma

alta taxa de multiplicação assexual, e é mantido por passagens intraperitoneais em roedores experimentais em laboratório (BARRETT et al., 1982).

1.5.1. Ciclo de vida

O ciclo de vida de *M. corti*, assim como as outras espécies do mesmo gênero, provavelmente inclui três hospedeiros (Figura 4). Entretanto, o ciclo de vida desta espécie tem uma particularidade entre os demais cestódeos, ser capaz de se reproduzir assexuadamente não somente no hospedeiro intermediário, mas também no hospedeiro definitivo carnívoro (ECKERT et al., 1969). Através do processo de apólice, a proglótide grávida, contendo o órgão pára-uterino cheio de oncosferas, é liberada pelo verme adulto no intestino delgado do hospedeiro definitivo, que a elimina com suas fezes. A continuidade do ciclo seria garantida através da ingestão da proglótide grávida, contendo as oncosferas, pelo primeiro hospedeiro intermediário. Após a eclosão, o embrião hexacanto penetraria na cavidade corpórea deste primeiro hospedeiro intermediário, onde se desenvolveria, presumidamente, em uma larva procercóide (PADGETT; BOYCE, 2005). A identidade do primeiro hospedeiro intermediário de *Mesocestoides* é incerta, sendo identificados ácaros oribatídeos do gênero *Trichoribates* infectados com larvas (SOLDATOVA, 1944), porem tentativas de infecção experimental dos mesmos ácaros e de outros invertebrados com ovos de diferentes espécies do gênero *Mesocestoides* não tiveram sucesso (LOOS-FRANK, 1991). Posteriormente foi sugerido que as formigas funcionariam como prováveis primeiros hospedeiros intermediários para *Mesocestoides* spp. (PADGETT; BOYCE, 2005), que foram infectadas pela ingestão de ovos do hospedeiro definitivo, porém não foi possível o desenvolvimento de tetratirídeos a partir de ratos alimentados com as formigas. Desta forma, a discussão sobre a identidade do primeiro hospedeiro intermediário, bem como, a presença de um ou mais hospedeiros intermediários no ciclo de vida de espécies do gênero *Mesocestoides* ainda persiste. A larva procercóide, com quatro ventosas, quando ingerida por um segundo hospedeiro intermediário, se transformaria no segundo estágio larval, o tetratirídeo. Este segundo hospedeiro intermediário é, presumivelmente, representado por espécies de anfíbios, répteis, aves ou mamíferos (SMYTH, 1987). O tetratirídeo

tem tamanho e forma variáveis, medindo de 0,5 a 2 mm de comprimento. É opaco, irregularmente pregueado e, na região anterior, possui um escólex bem desenvolvido, com quatro ventosas elípticas e musculares. Em infecções naturais, os tetratirídeos são encontrados livres nas cavidades peritoneal e/ou pleural; em massas císticas fixadas aos mesentéricos; e em vísceras, principalmente no fígado, onde estão, individualmente ou em grupos de vários indivíduos, envolvidos por tecidos fibrosos do hospedeiro (SPECHT; VOGE, 1965; TODD et al., 1978).

Tanto no hospedeiro intermediário natural como em camundongos ou ratos infectados experimentalmente, os tetratirídeos multiplicam-se assexuadamente, por cissiparidade anteroposterior (SPECHT; VOGE, 1965; ETGES, 1991). A cissiparidade envolve o desenvolvimento de ventosas supranumerárias, seguido de fissão do corpo a partir de sua extremidade anterior. A possível multiplicação assexual por brotamento é discutida para esta espécie, ainda não tendo sido esclarecido se os brotos desprendidos seriam capazes de originar tetratirídeos e/ou outra fase do desenvolvimento nos hospedeiros ou no cultivo *in vitro* (WHITFIELD; EVANS, 1983; MARKOSKI et al., 2003). No brotamento, seriam geradas protuberâncias laterais, ou brotos, na região posterior ao escólex. Os tetratirídeos podem também penetrar no fígado, nos pulmões ou outras vísceras de camundongos infectados experimentalmente, sendo igualmente encapsulados por fibrócitos do hospedeiro, em situação idêntica à observada em infecções naturais (SPECHT; VOGE, 1965; TODD et al., 1978). Ratos são menos suscetíveis que camundongos à infecção experimental, enquanto cobaias parecem ser resistentes à mesma (SPECHT; VOGE, 1965).

Mamíferos carnívoros e aves de rapina foram descritos como hospedeiros definitivos para espécies do gênero *Mesocestoides* (WILLIAMS; CONN, 1985).

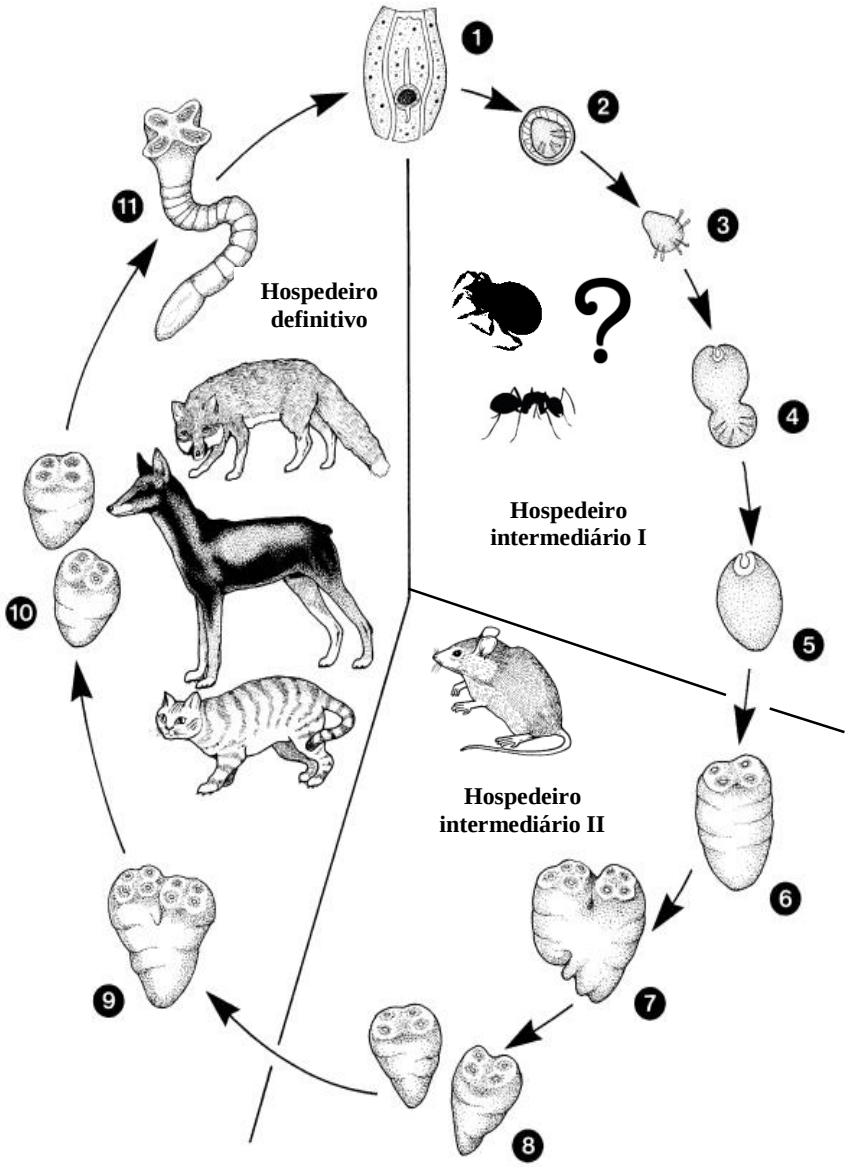

Figura 4 – Ciclo de vida de *Mesocestoides corti* – 1. O ciclo começa quando as proglótides que contém os ovos se desprendem do verme adulto e são liberadas no exterior nas fezes do hospedeiro. **2.** Ovo que contém a larva oncosfera que é ingerida pelo primeiro hospedeiro intermediario. **3-6.** Desenvolvimento no primeiro hospedeiro intermediário, que podem ser ácaros oribatídeos ou formigas. **7-9.** Desenvolvimento no segundo hospedeiro intermediário. As larvas, chamadas tetratirídeos (TTs, **7**) se alojam nas cavidades corporais de vários animais (repteis, anfíbios, aves ou mamíferos). A reprodução dos TTs acontece por fissão longitudinal (cissiparidade). **10-12.** O hospedeiro definitivo, mamíferos carnívoros, se infectam por meio da ingestão de tecidos contaminados com os TTs. Dentro do intestino delgado pode acontecer a reprodução assexuada do TT (**10**). Finalmente, o TT se desenvolve no verme adulto (**12**) com várias proglótides (atingindo até 40 cm de comprimento) que também podem penetrar outros órgãos ou cavidades corporais, onde se reproduz de forma assexuada (modificado de MEHLHORN, 2008).

Dentre os mamíferos, cães, gatos, gambás, vivérridos e raposas são os

hospedeiros definitivos mais recorrentes (TORRES et al., 2001; WICKER et al., 2016), infectando-se quando se alimentam de animais contendo tetratirídeos. Quando ingerida por um hospedeiro definitivo adequado, a larva pode penetrar na cavidade peritoneal e lá permanecer como tetratirídeo, ou, no intestino delgado, desenvolver-se como verme adulto (SPECHT; VOGE, 1965). O processo de desenvolvimento do tetratirídeo para verme adulto envolve crescimento, segmentação e proglotização do estróbilo. Durante a formação das proglótides, ocorre o processo de diferenciação sexual. O desenvolvimento das proglótides e órgãos sexuais ocorre no sentido anteroposterior. O verme adulto, hermafrodita, é capaz de autofecundação, com a cópula ocorrendo nas proglótides maduras (BARRETT et al., 1982). Após a fecundação, as genitálias masculina e feminina degeneram e são reabsorvidas restando, no segmento grávido, o órgão pára-uterino contendo oncosferas. Como em todos os membros da ordem Cyclophyllidea, não há abertura uterina, com as proglótides grávidas desprendendo-se do estróbilo à medida que completam o seu amadurecimento. A reprodução assexual do adulto supostamente ocorre por divisão longitudinal do escólex ou por brotamento lateral do estróbilo (SMYTH, 1987).

1.5.2. *M.corti* como modelo de estudo de desenvolvimento de cestódeos

M. corti constitui um modelo para o estudo da biologia do desenvolvimento de cestódeos. É de particular interesse devido a fácil manutenção de de constantes e grandes populações de tetratirídeos a través de passagens intraperitoneais sucessivas em camundongos e ratos (SPECHT; VOGE, 1965). O cultivo *in vitro* de tetratirídeos permite tanto o estudo da reprodução assexuada como a segmentação ou desenvolvimento estrobilar, dependendo das condições de cultivo utilizadas (THOMPSON et al., 1982; MARKOSKI et al., 2003). O desenvolvimento estrobilar é semelhante *in vitro* e *in vivo*, tornando-o um modelo ideal para o estudo deste processo, com a exceção de que ovos viáveis tem sido raramente documentados (BARRETT et al., 1982).

O sistema de cultivo *in vitro* de tetratirídeos foi alvo de vários estudos em busca de fatores indutores de estrobilização (BARRETT et al., 1982; SALDAÑA et al., 2001). Nosso grupo de pesquisa demonstrou que condições que mimetizam o microambiente do intestino no hospedeiro definitivo, como um

tratamento com protease (p. ex. tripsina, trombina, quimotripsina ou pepsina) e a alteração do pH e da fase gasosa dos cultivos, aumentam significativamente a taxa de estrobilização de tetratirídeos *in vitro* (MARKOSKI et al., 2003; ESPINOZA et al., 2005). Os componentes e mecanismos moleculares responsáveis pela estrobilização, entretanto, ainda estão por serem elucidados.

O estudo da expressão diferencial de genes no desenvolvimento estrobilar tem sido abordado no nosso grupo de pesquisa. Inicialmente, duas bibliotecas de cDNA, uma de tetratirídeos e outra de vermes adultos, foram construídas de acordo à técnica de RDA (*representational difference analysis*) para *M. corti*, e, através de microarranjo de DNA, foi possível identificar sequências conservadas de genes relacionados ao desenvolvimento de *M. corti* que apresentaram expressão diferencial entre a fase larval e a fase adulta (verme estrobilizado; BIZARRO et al., 2005). As principais diferenças obtidas na expressão foram metabolismo do ciclo celular, crescimento celular e outros processos celulares. Um resultado de particular interesse, foi a diferença da expressão de um conjunto de fatores de transcrição e reguladores da estrutura da cromatina, alguns dos quais foram identificados pela primeira vez em planárias. No trabalho foram sequenciados fragmentos de cDNA relacionados com os componentes da maquinaria molecular SWI/SNF, com ortólogos presentes em levedura, drosophila e na espécie humana, que recrutam atividades de remodelado da cromatina especificas a promotores homeóticos ou relacionados a mecanismos morfogênicos.

Em um trabalho posterior, Laschuk et al. (2011), realizaram um estudo das proteínas envolvidas nos eventos iniciais da estrobilização utilizando técnicas proteômicas e de eletroforese bidimensional. Especificamente, foi feita uma comparação entre tetratirídeos e vermes estrobilados, durante as primeiras 24 h da indução. Foi observado um enriquecimento, nos vermes induzidos a estrobilização, das proteínas com atividades chaperonas, síntese e renovação de proteínas.

Recentemente, *M. corti* tem sido utilizado como modelo para o estudo de células-tronco em helmintos parasitos, denominadas neoblastos. Em um trabalho inicial foi caracterizada a presença de células proliferativas durante o desenvolvimento de tetratirídeo a juvenil segmentado (KOZIOL et al., 2010) e posteriormente, foi desenvolvida uma estratégia para quantificação e isolamento

destas células proliferativas, utilizando citometria de fluxo e “cell sorting”, baseado no conteúdo de DNA das células (DOMÍNGUEZ et al., 2014).

Adicionalmente, *M. corti* tem sido proposto como um modelo importante para o ensaio e estudo de drogas anti-helmínticas e cestocidas, avaliando a atividade de uma diversidade de compostos, desde as de amplo uso como praziquantel e abendazole (MARKOSKI et al., 2006), assim como thymol (óleo essencial; MAGGIORE; ELISSONDO, 2014), e também de inibidores da enzima antioxidante tioredoxina glutationa transferase (PASQUET et al., 2015). Além disso, tem sido utilizado como modelo do estudo de secreção de fatores imunomoduladores na patogênese, por ter a vantagem, respeito por exemplo a *E. multilocularis*, de ser cultivável em condições livres de soro fetal (VENDELOVA et al., 2016).

Apesar dos progressos no estabelecimento do sistema de indução da estrobilização *in vitro*, e o estabelecimento do modelo de estudo de neoblastos em platelmintos, ainda não existe uma identificação completa dos genes envolvidos no processo de estrobilização. Recentemente, foi disponibilizada a versão rascunho (*draft*) do genoma de *M. corti* (50 Helminth Genomes Initiative, HOLROYD; SANCHEZ-FLORES, 2012), o que dá o ponto de partida para a obtenção de um quadro mais completo da expressão de genes no desenvolvimento deste parasito.

2. JUSTIFICATIVAS

Estudos sobre processos envolvidos na estrobilização de cestódeos, como a segmentação corporal e a diferenciação sexual, podem fornecer importantes subsídios para a elaboração de estratégias mais eficientes de prevenção e controle de cestodíases. Além disso, tais conhecimentos também são importantes para o esclarecimento de algumas questões filogenéticas pendentes relativas ao filo Platyhelminthes. Neste contexto, a caracterização de genes e proteínas envolvidas na estrobilização de platelmintos da classe Cestoda é fundamental para a viabilização de estudos comparativos mais amplos, a partir dos quais poderão ser melhor averiguados estas e outras relações suprafiléticas existentes entre platelmintos e outros grupos de metazoários.

Mesocestoides corti é um organismo modelo para o estudo de diversos aspectos da biologia dos cestódeos. Possui a vantagem de ter fácil manutenção em animais de experimentação, e de ser facilmente cultivável *in vitro*. O desenvolvimento estrobilar é semelhante ao de outros cestódeos tanto *in vitro* como *in vivo*, tornando-o um modelo ideal (BARRETT et al., 1982) para o estudo de aspectos dos mecanismos de desenvolvimento que não podem ser estudados em outros cestódeos, já seja por falta de material parasitário ou dificuldades para obter a forma adulta segmentada *in vitro*. Nesse sentido, no nosso grupo já foram estabelecidos e estão em pleno funcionamento os sistemas de manutenção de estoques *in vivo* de tetratirídeos de *M. corti*, em ratos e camundongos, e o sistema de cultivo *in vitro*, para indução do processo de estrobilização foi otimizado (MARKOSKI et al., 2003, 2006; ESPINOZA et al., 2005). Da mesma forma, foram realizados estudos transcritômicos e proteômicos para análise do padrão de expressão diferencial de um maior número de genes.

Embora a identificação destes genes do parasito tenha contribuído para entender alguns aspectos da biologia do desenvolvimento, a identificação não tem sido suficiente para revelar mecanismos moleculares envolvidos no controle de processos biológicos destes parasitos. Neste sentido, um estudo complementar e mais profundo do transcritoma permite a caracterização e a análise comparativa dos repertórios de genes expressos em tetratirídeos e vermes em processo de estrobilização. Os genes/proteínas identificados em este

trabalho poderão, posteriormente, ser caracterizados quanto aos respectivos padrões de expressão temporal e espacial e quanto a aspectos funcionais e a relevância na biologia do parasito. Além disso, a identificação do repertório de miRNAs expressos em *M. corti*, assim como a caracterização de seus padrões de expressão em diferentes estágios do ciclo vital deve de evidenciar repertórios de RNAs reguladores associados a determinados estágios ou processos de desenvolvimento do parasito. Por outra parte, ganhar conhecimento sobre os miRNAs expressos em uma espécie modelo de classe Cestoda fornece evidencia para analises comparativos dentro do grupo, e para estudos filogenéticos e evolutivos dentro do filo Platyhelminthes.

3. OBJETIVOS

O objetivo geral deste trabalho é a identificação e caracterização funcional de pequenos RNA regulatórios (miRNAs) e de genes/proteínas em dos estágios do desenvolvimento de *M. corti*, tetratirídeo e verme estrobilado, com o propósito de estudar mecanismos celulares e moleculares do processo de estrobilização na classe Cestoda.

O trabalho está dividido em duas partes (capítulos). Os objetivos específicos do capitulo 1 são:

- Caracterização do repertório de miRNAs de *M. corti* por RNA-seq e análise bioinformática.
- Estudo da expressão diferencial de miRNAs entre tetratirídeo e verme estrobilado.
- Análise da expressão dos miRNAs identificados.
- Estudo da conservação dos miRNAs de *M. corti*

Os objetivos específicos do capitulo 2 são:

- Caracterização dos genes diferencialmente expressos entre tetratirídeos e vermes estrobilados de *M.corti* por RNA-seq.
- Análise *in silico* das sequências obtidas, montagem do transcritoma, e identificação de genes expressos diferencialmente entre os dos estágios analisados do desenvolvimento de *M. corti*.
- Anotação funcional dos genes identificados como diferencialmente expressos entre ambos estágios.

4. CAPÍTULO 1 – Identificação e caracterização dos microRNAs de dos estágios do desenvolvimento do cestódeo parasito modelo *Mesocestoides corti*

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TB, HB e MR planejaram os experimentos e escreveram o manuscrito. TB realizou os experimentos. SE contribuiu com os experimentos. TB, NM e LK processaram os dados. TB e NM analisaram os dados. TB, NM, MC, LK, MR, HB discutiram os resultados. TB, AZ, MR e HB revisaram criticamente o manuscrito.

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Identification and profiling of microRNAs in two developmental stages of the model cestode parasite *Mesocestoides corti*

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ABSTRACT

MicroRNAs (miRNAs), a class of small non-coding RNAs, are key regulators of gene expression at post-transcriptional level and play essential roles in fundamental biological processes such as metabolism and development. The particular developmental characteristics of cestode parasites highlight the importance of studying miRNA gene regulation in these organisms. Here, we performed a comprehensive analysis of miRNAs in two developmental stages of the model cestode *Mesocestoides corti*. Using a high-throughput sequencing approach, we found transcriptional evidence of 42 miRNA loci in tetrathyridia larvae and strobilated worms. Tetrathyridium and strobilated worm-specific miRNAs were found, as well as differentialy expressed miRNAs between these developmental stages, suggesting miRNA regulation of stage-specific features. Moreover, it was shown that uridylation is a differential mechanism of post-transcriptional modification of *M. corti* miRNAs. The whole set of *M. corti* miRNAs represent 33 unique miRNA families, and confirm the remarkable loss of conserved miRNA families within platyhelminth parasites, reflecting their relatively low morphological complexity and high adaptation to parasitism. Overall, the presented results provide a valuable platform to studies aiming to identify and characterize novel miRNA-based molecular mechanisms of post-transcriptional gene regulation in cestodes, necessary for the elucidation of developmental aspects of the complex biology of these parasites.

1. Introduction

Tapeworms (phylum Platyhelminthes, class Cestoda) are obligate internal parasites of vertebrates that display a wide range of body forms and host associations. These parasites have evolved their own strategy for achieving enormous reproductive capability by increased fecundity through the serial repetition of their reproductive organs (proglottization). The strobilated condition, being strobilation the formation of strobila or segments [1], is believed to have evolved through a stepwise pattern in which proglottization and external segmentation were independent evolutionary events [2,3].

Despite several studies in the field of cestode biology, little is known about the molecular mechanisms underlying the developmental processes of these organisms, including strobilation. An increasing number of studies have been conducted to characterize genes and proteins involved in cestode development [4–7], but the current knowledge is still limited to define molecular pathways involved in the strobilation and other typical cestode developmental processes [8].

Mesocestoides corti is an endoparasitic platyhelminth used as experimental model to study the class Cestoda [9]. It is easily cultivated, is regarded as non-infective for humans, and lacks some of the experimental limitations associated with the work with other

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cestode parasites, including those concerning the availability of biological material and *in vitro* development capacity. The *in vitro M. corti* culture system allows monitoring of the whole strobilation process, from the larval metacestode stage (tetrathyridium) to the adult segmented worm, under controlled experimental conditions [10].

Small noncoding RNAs belonging to the microRNA (miRNA) class have emerged as important regulators of metazoan gene expression [11], being involved in many different biological processes, including development [12]. miRNAs are endogenous ~23 nt-long RNAs that play their gene-regulatory roles by pairing to target mRNAs to direct their post-transcriptional repression [13]. In the canonical biogenesis pathway, miRNAs are transcribed by RNA polymerase II into long primary miRNAs (pri-miRNAs) that are processed in the nucleus by a ribonuclease III (Drosha), generating the characteristic small hairpin known as pre-miRNA or precursor (~70 nt). In the cytoplasm, Dicer, another RNAse III protein, cleaves the pre-miRNA producing a short duplex of ~21–22 bp. In this duplex, the mature sequence is the functional one, while the complementary strand is called the miRNA star (miRNA*) strand [14]. This miRNA-miRNA* duplex is loaded into a RNA-induced silencing complex (RISC), in which the core RNA-binding protein is a member of the Argonaute family (AGO) [15]. Then, the star sequence is degraded and the mature sequence guides the AGO proteins to complementary mRNA sequences to repress their expression. The sequence comprising nucleotides 2–7 within the 5′-end of the mature miRNA is known as the 'seed' sequence, and is key for the miRNA-mRNA target interaction [16,17]. Mature miRNAs are subject to active regulation by editing, which could influence its stability or function. One mechanism of miRNA editing involves tailing (3′ addition of non-templated nucleotides, like uridine) and possibly induces degradation [18].

With the exception of some unicellular organisms, the key RISC machinery has been shown to be conserved across eukaryotes [19]. Within the phylum Platyhelminthes, the RISC machinery has been identified *in silico* in the free living turbellarian species [20], as well as in cestode and trematode parasite species [5,20–22], indicating that gene expression regulation by miRNAs is present in this phylum. Due to the increasingly affordable costs of next-generation sequencing (NGS), and from the rapid development of reliable quantitative methods for miRNA detection, data regarding miRNA profiles in parasitic helminths and host (tissue or circulating) miRNAs in response to helminth infection have expanded considerably in recent years [23]. For tapeworms, these studies have been largely limited to species of medical or economic importance such as *Echinococcus* spp. and *Taenia* spp. In fact, the first report of miRNAs in cestodes was performed for *E. granulosus* [24]. The miRNA population of *Echinococcus granulosus, Echinococcus multilocularis* and *Echinococcus canadensis* has been updated [25–27], but the data available for other tapeworms, such as *Taenia saginata* and *Taenia multiceps*, is still fragmented or absent [28,29]. Identification of the miRNAs expressed in *M. corti* may shed light on the mechanisms involved in the development of cestode parasites and provide information about the mechanism underlying the regulation of strobilation. In addition, gaining knowledge of the miRNA repertoire of a model species from the class Cestoda would provide support for comprehensive miRNA comparative analyses, and to phylogenetic and evolutionary studies within the phylum Platyhelminthes.

In this study, we performed for the first time a comprehensive analysis of miRNAs in the tetrathyridia larvae and strobilated worm of the model cestode *M. corti* using a high-throughput approach. A differential expression analysis was performed in order to identify regulated miRNAs between life cycle stages, which might suggest a role in maintaining the features of each developmental stage. The conservation analysis of miRNA families among related species was performed in order to address the reported loss of conserved miRNA families in platyhelminth parasites [30], which correlates to their low morphological complexity and high adaptation to parasitism. This study will provide valuable information for better understanding the complex biology of this parasite and other cestodes, and shed light on the molecular mechanisms underpinning the strobilation process.

2. Materials and methods

2.1. Parasite material

Mesocestoides corti larvae (tetrathyridia, TT) were maintained by alternate, serial passages in Wistar female rats and BALB/c female mice as previously described [9]. Experimental hosts were infected by intraperitoneal inoculation and, after 3 months, larvae were collected and used for experiments. Only TT from up to the third serial passage in mice were used for the experiments. Biological triplicates were used; each replicate produced using TT obtained from a single mouse host.

Larvae freshly collected from mice were washed six times in PBS plus streptomycin-penicillin (5 U/ml) and used immediately for culture. TT (n = 125) were kept in culture for 24 h in modified RPMI 1640 medium with 10% FBS (McRPMI) [9] at 37 °C and 5% CO_2. Then, larvae were washed three times with PBS, homogenized in Trizol reagent (Life Technologies) and stored at −80 °C until use for RNA extraction.

Strobilated worms (ST) were obtained from TT (n = 60) cultured under strobilation-inducing conditions as previously described [10]. Briefly, TT (n = 60) were incubated with trypsin 0.662% (w/v) in McRPMI for 24 h at 39 °C and 5% CO_2 for strobilation induction. Induced TT were then cultured in McRPMI without trypsin at 39 °C and 5% CO_2 until complete strobilation, which took around 10–12 days. The culture medium was replaced by fresh McRPMI every 48–72 h and the worms were inspected daily under a Zeiss inverted microscope to follow TT strobilation process and, eventually, to assess the percentage of those that reached the ST stage (which undergone both proglottization and segmentation). Only worm batches with a strobilation rate above 90% were used for experiments. ST were then washed three times with PBS, homogenized in Trizol reagent and stored at −80 °C until used for RNA extraction.

All experimental procedures for *in vivo* maintenance of *M. corti* TT in mouse and rat hosts were previously approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul (Project no. 25726).

2.2. Small RNA isolation

RNA preparations enriched in small RNAs (<200 nt, sRNA) were obtained from TT and ST. RNA extractions were carried out using Trizol according to the manufacturer's instructions and accordingly to [24]. The aqueous phase obtained after the organic phase separation step was enriched in sRNAs (RNAs < 200 nt) with the mirVana miRNA Isolation Kit (Ambion). Resulting RNA samples enriched in sRNAs were precipitated overnight at −20 °C with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vols of 100% ethanol, RNA was centrifuged at 14,000*g* for 30 min at 4 °C, air dried at room temperature, and resuspended in nuclease-free water. RNA concentration and integrity were determined using a Nanodrop 2000 (Thermo) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

2.3. sRNA library construction and sequencing

For each sRNA library construction, 100–200 ng of an RNA sample enriched in sRNAs were used as starting material. For each

parasite developmental stage, TT and ST, three libraries were constructed from independent samples (biological replicates). Libraries were constructed with the TruSeq Small RNA Sample Preparation Kit (Illumina) according to the manufacturerís instructions. Library size selection was performed in order to recover 18–30 nt-long sRNAs. The sRNA libraries were sequenced in an Illumina HiSeq 2000 sequencer in the same lane for 50 cycles. Library construction and sequencing were performed at Macrogen.

2.4. Source of genome assemblies and annotation

The *M. corti* draft genome assembly version 1.0.4 and CDS annotation was downloaded from the WormBase ParaSite (http://parasite.wormbase.org, Helminth Genomes Consortium). *M. corti* rRNA and tRNA sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov). Additional rRNA sequences from flatworms [31] were also retrieved from NCBI. Metazoan mature miRNA and precursor sequences were obtained from miRBase (release 21) [32]. *Echinococcus* spp. mature miRNA and precursor sequences were retrieved from two recently published high throughput analyses [25,26]. All annotated sequences, along with the new miRNA precursor sequences identified in this study, were used to construct an in-house database for sRNA library data classification (sRNA in-house database).

2.5. Bioinformatics analysis of M. corti small RNAs

Small RNA library data were pre-processed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) prior to its mapping to *M. corti* genome. After adapter trimming, low quality reads and reads <18 nt were removed to obtain clean reads. Identical clean reads were then collapsed into unique sequences with associated read counts. The processed reads were initially mapped to the *M. corti* genome with Bowtie (version 1.1.0) [33]. Then, to classify all sRNA library sequences as miRNAs, rRNA, tRNA, reads mapping in sense orientation to coding sequences (CDS/sense) or reads mapping in antisense orientation to CDS (CDS/antisense), all processed and mapped reads were analysed by BLASTN (with a <0.01 e-value cut off) against an in house database that included all miRNAs identified in this study (as described in "miRNA identification" section) and classified into the above mentioned categories. Reads with no match were grouped into a "no hit" category. A length distribution analysis of total mapped reads was also performed.

2.6. miRNA identification

The miRDeep2 (v 0.0.5) software package [34] was used to identify miRNAs from the sRNA libraries. Unique sequences were mapped to the *M. corti* genome (version 1.0.4) as described in [26]. For miRNA predictions with the miRDeep2 core algorithm, all metazoan mature miRNAs and precursor sequences (retrieved from the miRBase 21) were included. *E. granulosus* s.s., *E. canadensis* and *E. multilocularis* mature miRNA and precursor sequences were also used as input, as these cestode parasites are the closest phylogenetically related species to *M. corti* with available sequences from high throughput analyses [25–27]. The initial miRDeep2 output list of new miRNA precursors of each library was manually curated to generate a final high confidence set of miRNAs retaining only candidate new precursors with i) miRDeep2 score $\geq$5; ii) significant randfold *p*-value; iii) mature reads in the three replicate libraries of a given sample type, and; iv) presence of at least one star read. The secondary structures of putative precursors, with minimum free energy (MFE) less than −20 kcal/mol and with a mature miRNA located in the stem, were predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

The candidate new precursor sequences were then analysed using BLASTN (with a <0.01 e-value cut-off) against sets of rRNAs, tRNAs, and CDS. Predictions that overlapped with these sequence categories were removed.

miRNA names were assigned by miRBase database manager and will be uploaded in the next miRBase version.

2.7. miRNA annotation, identification of families and conservation analysis

To identify orthologous sequences of *M. corti* miRNAs, the full-length mature *M. corti* miRNA sequences were compared to the mature miRNA sequences present in miRBase 21, and with *Echinococcus* spp. mature miRNA sequences retrieved from [25–27] using SSEARCH (http://www.biology.wustl.edu/gcg/ssearch.html). Only sense matches were allowed, with a 70% nucleotide identity cut-off and according to the following seed match criteria: identical nucleotides 1–7 or 2–8 from the 5′ end of the mature miRNA. Those miRNAs that did not meet the above-mentioned requirements were considered new miRNAs. To identify *M. corti* miRNA families, all-against-all pairwise BLAST sequence alignments were performed and all sequences sharing the seed region (nt 1–7 or nt 2–8) were considered to belong to the same family. To analyse evolutionary conservation of *M. corti* miRNA families, mature miRNA sequences were compared to those present in miRBase 21 for selected phyla covering metazoan diversity: Cnidaria, Arthropoda, Annelida and Nematoda and to the subphylum Vertebrata using only a seed match criteria. The absence and presence of *M. corti* miRNA families in selected phyla were registered. In addition, for sequence conservation analysis, the full length mature miRNA sequences of conserved *M. corti* miRNAs identified in this study were aligned against a set of homologous full-length mature sequences of three selected model species: *Mus musculus*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, and against some related species of the Phylum Platyhelminthes: *Schmitdea mediterranea*, *Gyrodactylus salaris*, *Schistosoma mansoni, Schistosoma japonicum*, *E. granulosus* and *E. multilocularis*, using ClustalX [35].

2.8. miRNA abundance and differential expression analysis

For analysis of miRNA abundance levels, the read counts of each individual miRNA within a given sample were normalized to the total number of mature miRNA read counts in that sample [25,26]. Then, normalized miRNA read counts from biological replicates of the same sample type were averaged. A correlation analysis between independent biological replicates from each sample type (TT or ST) was performed. For this purpose, miRNA read counts within a replicate were plotted against miRNA read counts in the other replicate. All miRNAs identified in this study where considered for this analysis. Differential expression analysis of miRNAs between TT and ST was performed using DESeq [36] using raw reads as input. miRNAs expressed in both developmental stages that showed $-1\geq$ log2 fold change ≥ 1 and *p*-adjusted < 0.05 were considered differentially expressed. In addition, a miRNA was considered expressed in TT or ST stages when mature reads were identified in at least two biological replicates of each sample type.

Arm usage was determined by analysing product ratios of the 5′ (5p) and 3′ (3p) arms. When the minor product of a pre-miRNA showed $\geq$50% of reads with respect to the major product, miRNAs produced from both arms were considered mature miRNAs [37]. In the arm usage conservation analysis, data available in miRBase 21 was used for comparative purposes.

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2.9. Editing and post-transcriptional modification analysis

miRDeep2 outputs were manually inspected to determine the presence of editing and/or post-transcriptional modifications in mature miRNA sequences. For this analysis, only those sequences with at least 150 read counts [38] and representing more than 2.5% of the total reads for the corresponding mature miRNA in each library were considered. When more than one sequence with the same type of modification for a given miRNA reached these criteria, the read count numbers of the modified sequences were counted. A miRNA was considered to be under the effect of editing or post-transcriptional modifications when the read count number of the modified sequence(s) was ≥10% of the total read counts of the corresponding miRNA [39].

2.10. Experimental validation of miRNAs expression by poly(T) RT-qPCR

Selected miRNAs from TT and ST samples were experimentally validated by poly(T) reverse transcription followed by quantitative PCR (poly(T) RT-qPCR)-based expression analyses [40]. Prior to the reverse transcription reaction, 1 μg of the small RNA fraction was treated with DNase I (Sigma), and then polyadenylated with *Escherichia coli* Poly(A) Polymerase (New England Biolabs) for 60 min at 37 °C in a 20 μl reaction volume, according to the manufacturer's protocols. cDNA was synthesized from 100 ng of polyadenylated small RNAs from either TT or ST using SuperScript III Reverse Transcriptase (Life Technologies) and 0.5 μg of poly(T) adapter in a 20 μl reaction volume according to the manufacturer's protocol. Controls without reverse transcriptase were included for each sample. Reverse transcription was performed by using a 60 min at 50 °C–15 min at 70 °C program. For each RT-qPCR, 5 μl of diluted cDNA (1:100) was mixed with 0.5 μl of each primer (10 μM final concentration), 4 μl 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) and 10 μl sterile water in a final volume of 20 μl. RT-qPCRs were performed using a 7500 Fast Real-Time PCR system (Life Technologies). Cycling conditions were: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15, 60 °C for 20 s and 72 °C for 32 s. Dissociation curve analysis was carried out at the end of each qPCR run to verify amplification specificity for each target sequence.

The relative quantification miRNAs was performed using the $2^{-\Delta\Delta CT}$ method [41] using an endogenous constitutive control. Four biological replicates were used and no template controls were included for each primer pair. qPCR reactions were carried out in duplicate and the statistical significance assessed by the Student's *t*-test. The baseline and Cq were manually inspected and determined using the 7500 Software version 1.3.0 (Applied Biosystems). The primer sequences are listed in Supplementary Table 1.

To establish an endogenous control for qPCR experiments, the four most stable miRNAs between TT and ST samples according to sRNA-seq data (let-7-5p, miR-61-3p, miR-71-5p and miR-4989-3p) were selected along with one differentially expressed miRNA (miR-31-5p), used as an expression variability control. The RefFinder tool [42] (Supplementary Table 2) indicated miR-4989 as that with the most stable expression between biological replicates.

2.11. Data access

The small RNAseq data from this study have been deposited in NCBI's Gene Expression Omnibus [43] and are accessible through GEO Series accession number GSE85058 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85058).

3. Results

3.1. Small RNA sequencing

sRNA libraries were generated from the tetrathyridia larvae (non strobilated) and strobilated worm stages (TT and ST samples, respectively) of *M. corti*'s life cycle in order to define the miRNA repertoire of each stage and to identify miRNAs involved in *M. corti* strobilation. Three biological replicates from each stage (TT or ST) were sequenced, and the sequencing results are summarized in Table 1. The total numbers of raw reads ranged from 31.7 to 35.9 million per sample, and, after removal of low quality reads and adapter sequences, the numbers of clean reads ranged from 16.7 to 24.9 million (50–74% of total reads) per sample. Percentages of reads mapping to the *M. corti* genome ranged from 73.5 to 88.1%, for TT samples, and from 67.9% to 83.7%, for ST samples.

Sequence classification (Fig. 1A) of mapped reads showed that TT sRNA libraries contained, on average, 56% of miRNAs, 6% of rRNA, 3% of reads mapping to CDS/sense, 3% of reads mapping to CDS/antisense, 2% of tRNA, and 29% of sequences with no hit. ST sRNA libraries, in turn, contained on average 74% of miRNAs, 4% of rRNA, 3% of reads mapping to CDS/sense, 3% of reads mapping to CDS/antisense, 3% of tRNA, and 13% of sequences with no hit.

Length distribution analysis (Fig. 1B) showed that the sRNA profile of total mapped reads was similar for both TT and ST samples. The most frequent read length was 22 nt, as expected for Dicer-derived products, thus supporting the presence of mature miRNA molecules in *M. corti*.

3.2. Identification of conserved and new miRNAs from M. corti

After the removal of rRNAs, tRNAs, and reads that mapped to CDS, the remaining reads were used to search for both conserved and new miRNAs using miRDeep2. The conserved and candidate new mature miRNAs that were identified in *M. corti* samples are shown in Table 2.

We considered that both arms of the same hairpin produced two mature miRNAs when the number of read counts of the minor product represented 50% or more of the read counts of the major product, originated from the opposite arm. By doing this, we observed that three miRNAs precursors showed expression from both arms in TT samples, namely mir-153, mir-190, and mir-12068, while other two, mir-153 and mir-12068 showed expression from both arms in ST samples (Supplementary Table 3).

The repertoire of *M. corti* miRNAs is encoded by 42 loci (37 conserved and 5 new miRNAs, Table 2), grouped in 33 families. Out of these miRNAs, 36 conserved and 6 new mature miRNAs were identified in TT samples, while 38 conserved and 5 new mature miRNAs were identified in ST samples. Therefore, the total number of mature miRNAs is 42 in TT samples and 43 in ST samples, while the number of pre-miRNAs is 39 and 41 for TT and ST samples, respectively (Table 2, Supplementary Table 4).

Based on stage specific expression pattern, one new miRNA, miR-12071, was considered TT-specific, and 3 conserved miRNAs, namely miR-2b, miR-7a, and miR-3479b, were considered ST-specific.

Regarding the features of the *M. corti* miRNAs, the mature miRNA sequences from both stages had lengths between ~22-23 nt and most of them (around 73% for both stages) started with an uracil, while precursor sequences had a medium length of 67 nt (Supplementary Table 4). In addition, they present high percentage of identity with mature and precursor sequences of the related cestode parasite *E. multilocularis* (Table 2). Furthermore, all miRNAs identified form stable hairpin structures (minimum free energy ≤ −17 kcal/mol), which is essential for the processing of pre-miRNA transcripts into mature miRNAs (Supplementary Fig. 1).

Table 1
–Summary of sequencing results obtained from small RNA libraries of *Mesocestoides corti* tetrathyridia (TT) and strobilated worms (ST).

	TT samples			ST samples		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Raw reads	33,477,647	33,263,853	33,391,571	31,707,701	32,548,060	35,961,076
Clean reads	16,703,698	17,923,438	24,910,387	21,969,990	23,133,658	20,757,428
Clean reads (≥18nt)	8,743,910	7,947,066	13,640,395	14,232,891	15,785,899	12,573,010
Mapped reads	6,663,322	5,844,848	12,018,162	11,841,006	13,213,856	8,542,583
Percentage of mapped reads[a]	76.2%	73.5%	88.1%	83.2%	83.7%	67.9%
Unique reads	1,302,902	1,286,675	1,656,001	1,309,141	1,383,638	1,227,070

[a] Relative to clean reads.

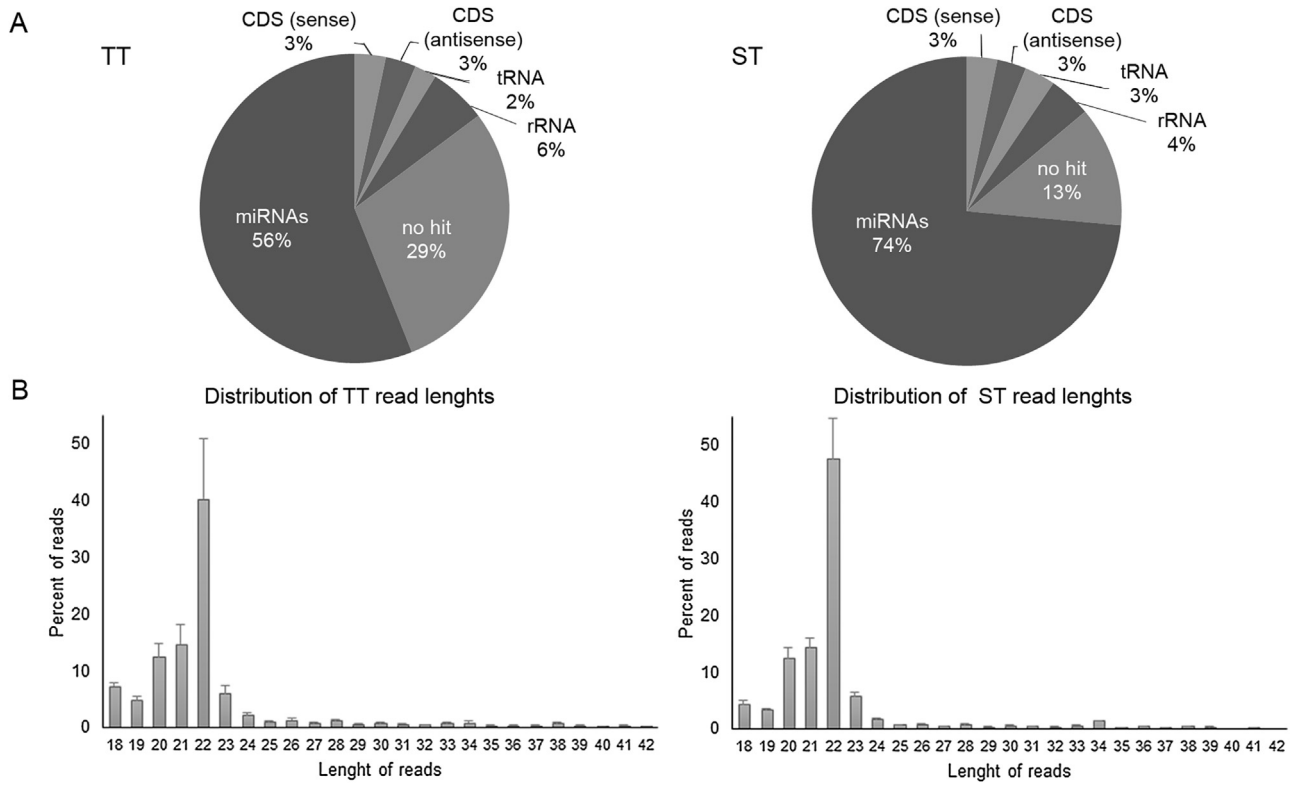

Fig. 1. – Small RNA library composition and length profiles of *Mesocestoides corti* sequencing data. A) Classification of small RNAs of *Mesocestoides corti* tetrathyridia (TT) and strobilated worm (ST): miRNA: microRNAs, rRNA: ribosomal RNAs, tRNA: transfer RNAs, CDS: protein coding sequences, no hit correspond to those not annotated. B) Length distribution analysis of sequenced reads of TT and ST samples; the average proportion in each replicate is displayed.

Seven conserved *M. corti* miRNAs with particularly long hairpins: bantam, mir-7b, mir-36b, mir-87, mir-96, mir-277 and miR-12066 could only be identified after changing the miRDeep2 algorithmic parameters for the excision of hairpins. Star sequence reads were identified for almost all *M. corti* miRNAs adding confidence to the miRNA predictions. Let-7 was one of the first discovered miRNAs and has been described as highly expressed in other platyhelminth parasites [25–27,44,45] indicating the importance of this miRNA in the parasite biology and metabolism. In this work, we detected a high number of reads corresponding to let-7 mature sequence (15–19% of all miRNA reads).The *M. corti* let-7 (mco-let-7) precursor sequence was manually detected by a BLAST search against *M. corti* genome using the *E. multilocularis* let-7 precursor sequence as query. Fig. 2A shows the predicted secondary structure for the identified putative mco-let-7. Its stem-loop structure, characteristic of pre-miRNAs, further confirmed the presence of a let-7 precursor in *M. corti*.

To investigate the possible occurrence of miRNA gene clustering, the genomic arrangement of the *M. corti* identified miRNAs was assessed (Supplementary Table 4). Seven miRNAs were found to be organized in three clusters: mir-1, mir-2b, mir-2c, mir-71, mir-133, mir-277 and mir-4989. Two clusters are formed by two miRNAs (mir-1/133, and mir-277/4989), and one cluster is composed by three miRNAs (mir-71/2c/2b). Fig. 2B and C shows the predicted secondary structures of the mir-277/4989 and mir-71/2c/2b clusters. The predicted secondary structure of the mir-277/4989 cluster forms two main hairpin stem-loop structures and comprises a 208 bp region (contig0000263), while the predicted secondary structure of the mir-71/2c/2b cluster forms the three hairpin stem-loop structure and comprises a 273 bp region (scaffold0000096). In the mir-1/133 cluster, on the other hand, the two encoded miRNAs are located approximately 18 kb apart from each other.

3.3. Expression profiles of *M. corti* TT and ST miRNAs

The TT and ST miRNAs abundance was assessed by counting the relative number of sequenced reads in each sample (Table 2). The top five predominant miRNAs in TT and in ST samples are shown in Fig. 3. Altogether, they accounted for ~90% of the total miRNA reads in each sample, and, interestingly, they were the same for both life stages (bantam-3p, let-7-5p, miR-10-5p, miR-71-5p, and miR-4989-3p). From these, miR-10 and let-7 accounted for ~75% of the

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Table 2
–Catalog of mature microRNAs (miRNAs) of *Mesocestoides corti* TT and ST life cycle stages.

microRNA[a]	miRNA mature sequence[b]	Normalised read counts[c]		miRNA length (nt)	miRNA family[d]	*E. multilocularis*	
		TT	ST			% Identity mature	% Identity precursor
mco-bantam-3p	UGAGAUCGCGAUUAAAGCUGAU	50,967	56,167	22	bantam	95.5	68.9
mco-let-7-5p	UGAGGUAGUGUUUCGAAUGUCU	188,408	150,271	22	let-7	100.0	96.0
mco-miR-1-3p	UGGAAUGUUGUGAAGUAUGU	88	55	20	mir-1	100.0	87.1
mco-miR-2a-3p	UAUCACAGCCCCGCUUGGAACU	2566	3835	22	mir-2	95.0	76.4
mco-miR-2b-3p	UAUCACAGCCCUGCUUGGGAC	ND	8030	21	mir-2	100.0	81.8
mco-miR-2c-3p	UCACAGCCAAUAUUGAUGAAC	8792	6832	21	mir-2	100.0	89.1
mco-miR-7a-5p	UGGAAGACUGGUGAUAUGUUGU	ND	6	22	mir-7	100.0	79.4
mco-miR-7b-3p	CAACUGUCACGGUCUUCCAAGU	44	45	22	mir-7	NA	82.1
mco-miR-9-5p	UCUUUGGUUAUCUAGCUGUGU	3984	1943	21	mir-9	100.0	83.1
mco-miR-10-5p	CACCCUGUAGACCCGAGUUUGA	535,863	604,117	22	mir-10	100.0	81.8
mco-miR-31-5p	UGGCAAGAUACUGGCGAAGCUGA	2	60	23	mir-31	100.0	80.0
mco-miR-36a-3p	CCACCGGGUAGACAUUCAUCCACU	98	12	24	mir-36	94.4	77.8
mco-miR-36b-3p	UCACCGGGUAGCGAUUACGCUU	38	14	22	mir-36	86.4	67.4
mco-miR-61-3p	UGACUAGAAAGUGCACUCACAUC	15,194	10,437	23	mir-279	95.7	78.9
mco-miR-71-5p	UGAAAGACGAUGGUAGUGAGAU	73,359	63,320	22	mir-71	100.0	73.3
mco-miR-87-3p	GUGAGCAAAGUUUCAGGUGUGC	9776	7898	22	mir-87	100.0	79.8
mco-miR-96-5p	AUUGGCACUUUUGGAAUUGU	1882	2073	20	mir-96	100.0	72.2
mco-miR-124a-3p	UAAGGCACGCGGUGAAUGCC	146	131	20	mir-124	100.0	68.5
mco-miR-124b-3p	UAAGGCACGCGGUGAAUAC	153	175	19	mir-124	100.0	89.1
mco-miR-125-5p	UCCCUGAGACCCUAGAGUUGUC	3465	5500	22	mir-125	100.0	82.4
mco-miR-133-3p	UUGGUCCCCAUUAACCAGCCGCU	58	32	23	mir-133	100.0	82.8
mco-miR-153-5p	AUGCUUAUGUGACGUGCACUC	269	165	21	mir-153	ND	81.1
mco-miR-153-3p	UUGCAUAGUCUCAUAAGUGCCA	359	253	22	mir-153	100.0	81.1
mco-miR-184-3p	GGGACGGAAGUCUGAAAGGGUUU	2255	1541	23	mir-184	95.5	91.1
mco-miR-190-5p	AGAUAUGUUUGGGUUACUUGGUG	1798	2545	23	mir-190	100.0	80.0
mco-miR-190-3p	CCAGUGACCGAACAUAUUCACA	1362	ND	22	mir-190	ND	80.0
mco-miR-219-5p	UGAUUGUCCAUUCGCAUUUCUUG	6439	3821	23	mir-219	100.0	86.1
mco-miR-277-3p	UAAAUGCAUUUUCUGGCCCGUAA	10,197	5557	23	mir-277	100.0	83.3
mco-miR-277b-3p	UAAAUGCAAAAUAUCUGGUUAUG	330	187	23	mir-277	100.0	87.7
mco-miR-281-3p	UGUCAUGGAGUUGCUCUCUAU	2395	1849	21	mir-281	100.0	94.3
mco-miR-307-3p	UCACAACCUACUUGAUUGAGGGG	3459	3761	23	mir-67	100.0	74.7
mco-miR-745-3p	UGCUGCCUGGUAAGAGCUGUGA	4279	2623	22	mir-22	100.0	86.0
mco-miR-2162-3p	UAUUAUGCAACAUUUCACUCA	856	612	21	mir-1993	95.0	73.7
mco-miR-3479a-3p	UAUUGCACUUUCUUUCGCCUUC	5468	2410	22	mir-92	90.9	84.2
mco-miR-3479b-3p	GAUUGCACUACCCAUCGCCCUCU	ND	188	23	mir-92	95.5	73.5
mco-miR-4989-3p	AAAAUGCACCAACUAUCUGAGA	62,840	50,555	22	mir-277	100.0	80.0
mco-miR−10293-3p[e]	UAAUUCGAGUCAACAGGGUCGUU	22	5	23	mir−10293[d]	100.0	86.2
mco-miR-12065-3p	UGUGCGUAGUUGCAAUGACCU	389	148	21	mir-210	ND	ND
mco-miR-12066-3p	UUGUGCGUCGUUUCAGUGACCGACA	1828	1434	25	mir-210	ND	ND
mco-miR-12067-3p	UAUGGAUAAGGCGUAUGACUCC	122	97	22	novel	NA	NA
mco-miR-12068-5p	UACCCUCACCGGUCGUGCAAGG	50	54	22	novel	NA	NA
mco-miR-12068-3p	UUGUACUACUGGCGAGGGUAGU	42	56	22	novel	NA	NA
mco-miR-12069-3p	UGUACUACAACAGGCCGUGGCU	277	130	22	novel	NA	NA
mco-miR-12070-3p	GCUACCGAAACGACUUAACUU	63	35	21	novel	NA	NA
mco-miR-12071-5p	GAGGGCCAUUGACAGUCGUUG	18	ND	21	novel	NA	NA

ND, not detected; NA, not aplicable.

[a] miRNA names were assigned by the miRBase database manager and will be uploaded in the next version.

[b] The canonical (100% identical to the reference genome) most frequent read from both biological replicates is reported. When the minor product of a precursor (pre)-miRNA showed a read count number ≥50% with respect to the major product, it was considered as mature miRNA.

[c] miRNA read counts were normalized to the total number of mature miRNAs in each library, multiplied per 1×10^6 and averaged between biological replicates.

[d] Novel families were defined for new miRNAs identified, which didnít show seed conservation.

[e] First reported in *Echinococcus multilocularis* and *E. canadensis* by Cucher et al. (2015, supplementary table S7). The names emu-miR-10293-3p and egr- miR-10293-3p were assigned by the miRBase database manager and will be uploaded in the next version.

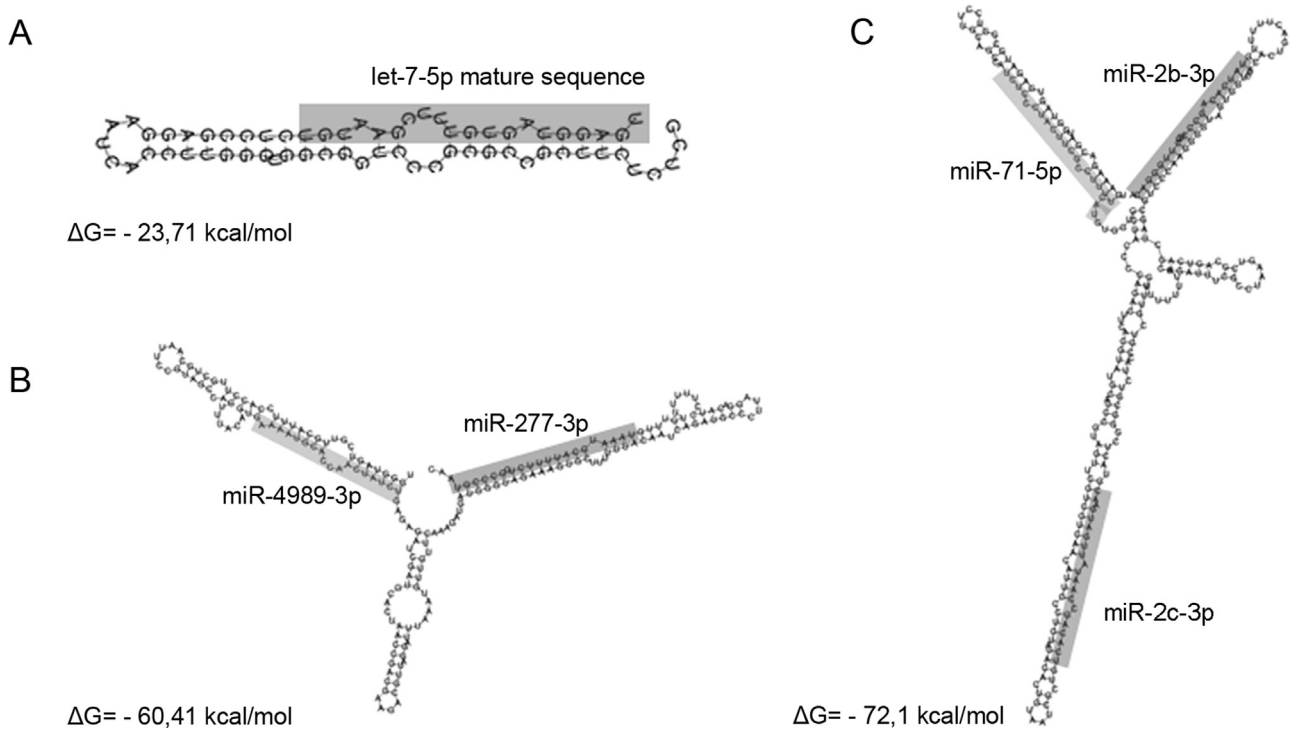

Fig. 2. – Predicted secondary structure of *Mesocestoides corti* miRNA precursors. A) *Mesocestoides corti* miRNA-let-7 precursor secondary structure predicted with RNA fold. B and C) The predicted secondary structures of the *M. corti* miRNA-4989/277 (B) and miRNA-71/2b/2c (C) clusters. The corresponding mature miRNA sequences are highlighted in grey and the free energy (ΔG) is shown.

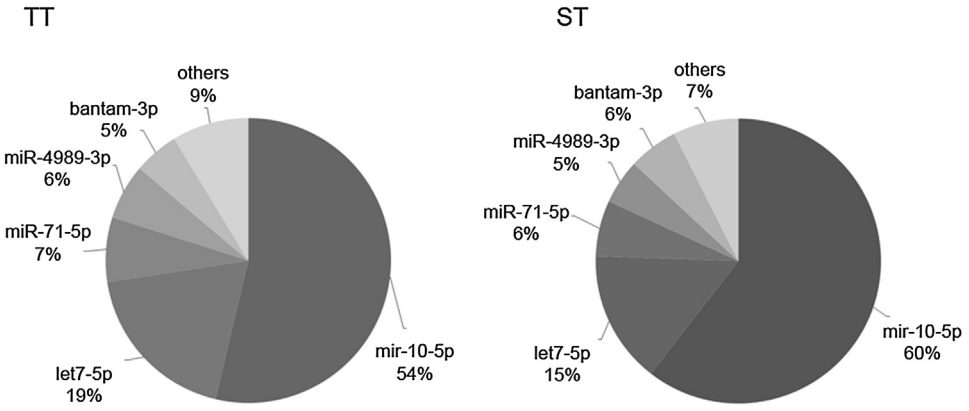

Fig. 3. – Circle chart with the percentages of the five most abundant miRNAs in the two life-stages of *Mesocestoides corti*: tetrathyridia (TT) and strobilated worm (ST). Results are shown as average percentages (± S.D.) of biological replicates.

total miRNA expression in both TT and ST samples. The high expression levels of these five miRNAs in two different life stages suggests their role in constitutive functions, highly relevant for parasite's survival and development. The miRNAs found in *M. corti* TT and ST samples are expressed at very different levels. For instance, most of the miRNAs identified: 83% (35/42) and 86% (37/43) in TT and ST samples, respectively, showed low expression levels, accounting for less than 1% (<100 thousand reads) of the expression levels detected (Table 2, Supplementary Table 4).

Interestingly, some of the miRNAs grouped in clusters showed an asymmetrical transcription pattern. For instance, miR-71-5p is one of the top five most expressed miRNAs, while miR-2b-3p and miR-2c-3p are at least ten fold less expressed (Supplementary Table 4). A very similar pattern is observed regarding the mir-277/4989 cluster, were miR-4989-3p is six fold more expressed than miR-277-3p (Supplementary Table 4). This might suggest the presence of a post-transcriptional mechanism affecting the stability of mature miRNAs organized in the same cluster, since two of *M. corti* miRNA clusters comprises a small genomic region and could be part of a single transcriptional unit.

We also analysed whether each mature miRNA product preferentially originated from one arm of the hairpin. As shown in Supplementary Table 3, around 70% of the mature miRNAs in *M. corti* are processed from the 3p arm of the hairpin, while around 23% are processed from the 5p arm of the hairpin. Three pre-miRNAs precursors showed expression from both arms, namely mir-153, mir190, and mir-12068 in TT samples, and only mir-153 and mir-12068 in ST samples (Supplementary Table 3).

The expression of 6 mature miRNAs, namely bantam-3p, let-7-5p, miR-7a-5p, miR-10-5p, miR-87-3p and miR-4989-3p, was confirmed by RT-qPCR in TT and ST samples (Supplementary Table 5). Furthermore, the high expression levels of let-7-5p and miR-10-

5p detected in the sRNA-seq data were validated in both ST and TT samples.

3.4. Differential expression analysis between TT and ST miRNAs

After the analysis of miRNA abundance, we conducted a comparative expression analysis for those miRNAs identified in both TT and ST samples. First, a correlation analysis was performed between independent biological replicates from each sample type, which showed high reproducibility of the data (Supplementary Fig. 2) thus enabling differential expression analysis. Then, differential expression of miRNAs between TT and ST was assessed using DESeq algorithm (Fig. 4A). We found that mi*R*-36b-3p and the candidate miR-12065-3p were significantly up-regulated in TT, while miR-2a-3p, mi*R*-31-5p, miR-125-5p, and miR-190-5p were up-regulated in ST samples. mi*R*-36b-3p showed the most noticeable up-regulation in the TT stage, with its expression approximately 4-fold higher than in the ST stage, while mi*R*-31-5p presented the most noticeable up-regulation in the ST stage, with an almost 32-fold difference in comparison to that in the TT stage. We also identified some stage-specific miRNAs, expressed only in one developmental stage, namely miR-12071-5p, specific for TT (Fig. 4B), and miR-2b-3p, miR-7a-5p, and mi*R*-3479b-3p, specific for ST (Fig. 4C). These stage-specific miRNAs could be involved in regulating specific features, of the developmental stage in which they are expressed. However, the number of miR-7a-5p and miR-12071-5p reads was very low, implying that stage specificity should be confirmed with higher coverage approach.

Differential expressions of two stage biased miRNAs in TT samples (mi*R*-36b-3p and miR-12065-3p) and three selected stage biased miRNAs in ST samples (mi*R*-31-5p, miR-125-5p, miR-190-5p) was validated by RT-qPCR (Supplementary Table 6). Among them, two miRNAs: mi*R*-31-5p and miR-125-5p were concordant between RNA-seq and RT-qPCR, thus confirming the RNA-seq differential expression data.

3.5. miRNA editing

In order to identify miRNA isoforms (isomiRs), generated by the non-template additions in *M. corti* miRNAs, the presence of uridine (U) at the 3′ end was queried in our datasets. miRNA processing by addition of a single 3p terminal U was found for 13 TT miRNAs and for 15 ST miRNAs (Fig. 5, and Supplementary Table 7). The isomiRs identified in this study were detected in all biological replicates of each sample, and, when identified in both TT and ST samples (11 miRNAs), they corresponded to the same sequence.

In most cases, for both TT (11 out of 13) and ST (13 out of 15), the unmodified form was more abundant. For miR-184-3p and miR-7b-3p, however, the isomer-U presented the same abundance (or even slightly higher) that the unmodified form in both life stages. miR-184-3p presented isomer-U abundances of 53% and 49% in TT and ST, respectively, while miR-7b-3p presented isomer-U abundances of 56% and 46% in TT and ST, respectively. Interestingly, there were differential uridylation patterns between life stages, mi*R*-36b-3p (20%) uridylated only in TT, and mi*R*-31-5p (11%) uridylated only in ST samples. Furthermore, different levels of uridylation were also observed among miRNAs organized in cluster. In one of the identified *M. corti* miRNA clusters (mir-71/2c/2b) only miR-2b-3p was uridylated. In the mir-1/133 cluster, only miR-133-3p was uridylated. Such differential patterns of uridylation are suggest that a possible tailing mechanism of post-transcriptional regulation through the parasite's life cycle is present in *M.* corti.

Primary miRNA editing by adenosine modification into inosine was also investigated in our *M. corti* data sets. We found no significant evidence of adenosine modification to iosine in TT or ST samples.

3.6. Sequence conservation analysis of M. corti miRNAs

All 45 *M. corti* miRNAs identified in this study grouped in 33 miRNA families, based in a seed match criteria. In order to identify conserved and new miRNA families present in *M. corti*, we compared the mature miRNA sequences identified in this study against all miRBase v21 database using only a seed match criteria. This analysis allowed the identification of 28 conserved and 5 new *M. corti* miRNAs families. From the conserved miRNA families, one present in all eumetazoans, 18 in bilaterians, 8 in protostomes and one so far only described in the genus *Echinococcus* (miR-10293).The conserved *M. corti* miRNAs families, shared with selected phyla Cnidaria, Nematoda, Arthropoda, Annelida and with the subphylum Vertebrata are shown in Fig. 6. From the 33 miRNA families identified in *M. corti*, 7 families presented multiple members. The largest ones were the mir-2 family, integrated by miR-2a, miR-2b, and miR-2c, and the mir-277 family, integrated by miR-277, miR-277b and miR-4989. The other *M. corti* miRNA families with multiple members were mir-7 (miR-7a, miR-7b), mir-36 (mi*R*-36a and 36b), mir-124 (miR-124a and 124b), mir-92 (mi*R*-3479a and 3479b) and mir-210 (miR-12065 and miR-12066).

Among the five *M. corti* candidate new miRNAs identified, only one, miR-12067 shared seed sequence (but not 70% of nucleotide identity in the full sequence) with a conserved miRNA, mir-1262, reported in *Homo sapiens* (Fig. 7A). As mentioned before, we identified two members of the mir-210 family (miR-12065 and miR-12066), already described in *S. mediterranea* (phylum Platyhelminthes) and in vertebrates (Fig. 7A), but so far never described in cestode or trematode platyhelminths.

The degree of conservation of *M. corti* miRNAs belonging to conserved families, was assessed comparing their full-length mature sequences against selected species present in miRBase v21, with seed match and ≥70% homology criteria. This comparative analysis was performed against three model species, namely *M. musculus*, *D. melanogaster*, and *C. elegans*, and against some related species of the Phylum Platyhelminthes, namely *S. mediterranea*, *G. salaris*, *S. mansoni, S. japonicum*, *E. granulosus* and *E. multilocularis* (Supplementary Fig. 3). As expected, the majority of the conserved *M. corti* miRNAs (36) have homologs with ≥70% identity in the cestodes *E. multilocularis* and *E. granulosus* (34/36; 94%). On the other hand, the majority of *M. corti* miRNAs (31/36; 86%) have homologs with ≥70% identity in the free living *S. mediterranea* (Turbellaria). Interestingly, a lower number of *M. corti* miRNAs (26 and 23, respectively) are shared with the ectoparasite *G. salaris* (Monogenea), and *S. japonicum* (Trematoda), which also belong to the Neodermata clade. Additionally, 19 (53%), 24 (66%) and 16 (44%) of *M. corti* miRNAs have homologs in *C. elegans* (Nematoda), *D. melanogaster* (Arthropoda), and *M. musculus* (Chordata), respectively. Among all the species analysed, a high conservation was observed among the full-length mature sequences of miR-71 orthologs (Fig. 7B), suggesting functional conservation. On the other hand, some miRNAs, like let-7 and miR-125, seem to be conserved within cestodes, but are highly divergent from their counterparts from other organisms. Other miRNAs, like bantam, showed more conservation among platyhelminth parasites, being more divergent from the other analysed species.

4. Discussion

In this work, we provided experimental evidence of miRNA expression in *M. corti* describing, for the first time, miRNAs reper-

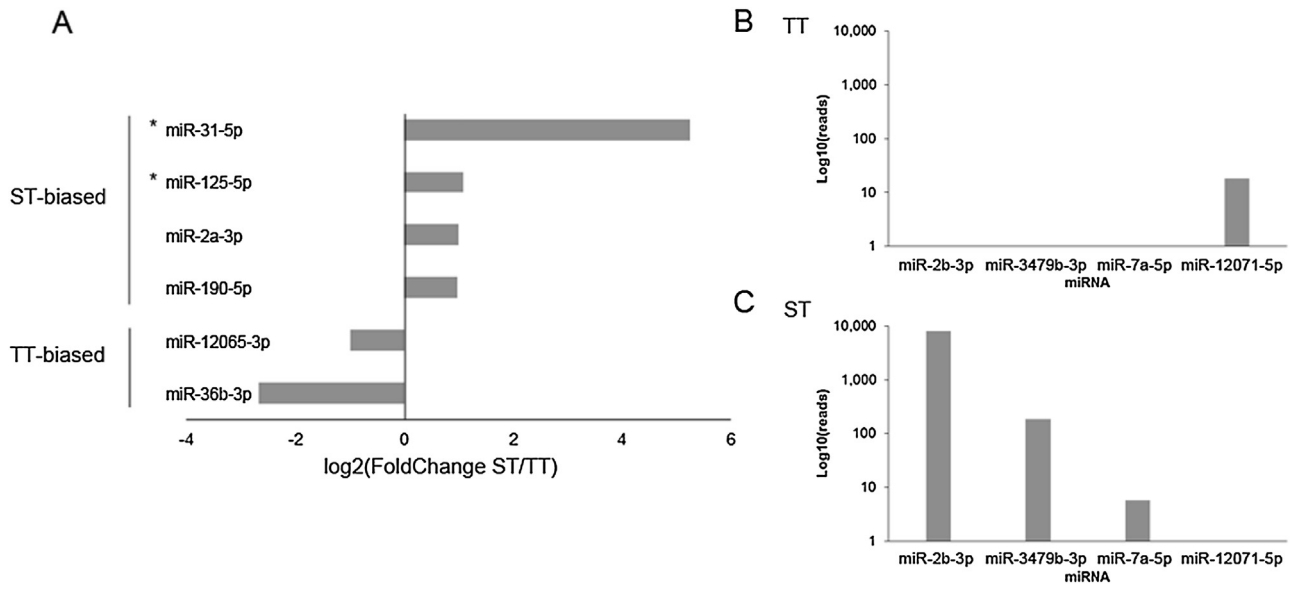

Fig. 4. – Differential expression of microRNAs between tetrathyridia (TT) and strobilated worm (ST) of *Mesocestoides corti*. A) Fold change analysis using DESeq algorithm. miRNA with Log_2 fold change $\geq \pm 1$ and *p*-adjusted < 0.05 are displayed; an asterisk (*) marks stage-biased microRNAs validated by real time PCR (*p*-value < 0.0001, based on *t*-test). B and C) Normalized expression levels of miRNAs detected exclusively in TT (B) and ST samples (C); read counts of each miRNA were normalized to the total number of mature miRNA read counts in that sample.

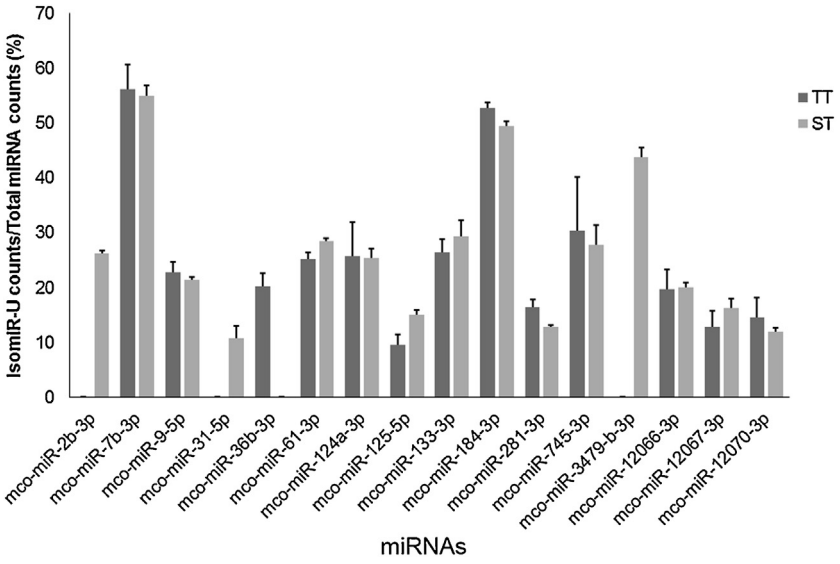

Fig. 5. – IsomiR-U expression profiles in *Mesocestoides corti*. Results are shown as average percentages (±S.D.) of biological replicates. TT (Tetrathyridia), ST (Strobilated worm).

toires of the tetrathyridia larvae and of the strobilated worm, two developmental stages of this model cestode parasite.

miRNAs are the most abundant type of small RNAs in *M. corti*, and the percentages of reads corresponding to miRNAs in TT and ST (56 and 76%, respectively), are in agreement with previous high-throughput analyses in *Echinococcus* [25,26]. We hypothesize that the fraction of miRNAs is larger in ST samples as a reflex of the adult increased morphological and physiological complexity, in comparison to the mostly undifferentiated TT [46–48], since the other assigned categories did not show significant differences between TT and ST samples whatsoever. In agreement with this, a higher fraction of miRNAs was identified in protoscoleces (pre-adult stage) than in cyst wall (larval stage) in *E. canadensis* [26].

The miRNAs identified for *M. corti* in this study share some features with other related organisms, such as a similar sequence length (for both pre-miRNAs and mature miRNAs), a high percentage of mature miRNAs sequences beginning with uracil (U), a bias towards 3′ arm processing, and miRNA tailing, conserved among parasitic platyhelminths [25,44,45] as well as in nematodes [49]. Some of the uridylated isomiRs found in *M. corti*, namely miR-2b, miR-9, miR-61, miR-124a, miR-125, miR-184, miR-745, and miR-3479b, were also found in *E. canadensis* and *E. multilocularis* [25]. This conservation pattern suggests that regulation of these miRNAs by uridylation could be a mechanism shared by cestodes to regulated miRNA stability and expression.

The number of new miRNAs reported in different platyhelminths is quite variable, and may vary as function the stringency of the pipelines of analysis. The *M. corti* sRNA-seq data analysis yielded a smaller number of new miRNAs (6) in comparison to those reported for *E. granulosus* (94), *S. japonicum* (38) and *S. mediterranea* (48) [27,45,50]. This lower number of new miRNAs in the *M. corti* repertoire could be due, at least in part, to the more strin-

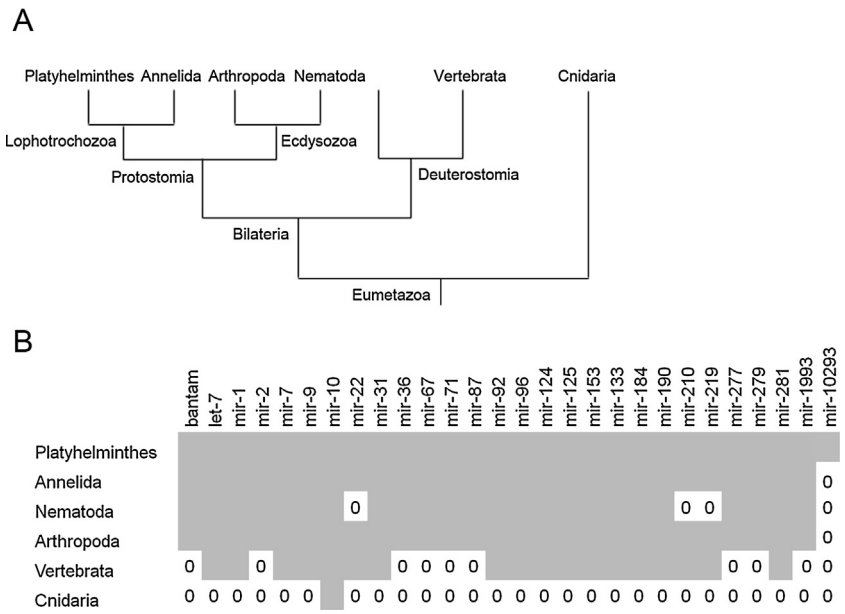

Fig. 6. – Conservation of *Mesocestoides corti* miRNA families. A) Simplified tree of Eumetazoa. B) Conservation analysis between phyla. miRNA belonging to phyla Annelida, Nematoda, Arthropoda, Cnidaria and subclass Vertebrata were compared using seed match criteria. Light grey rectangles show presence of the miRNA family in the clade, 0 represents absence of the miRNA family.

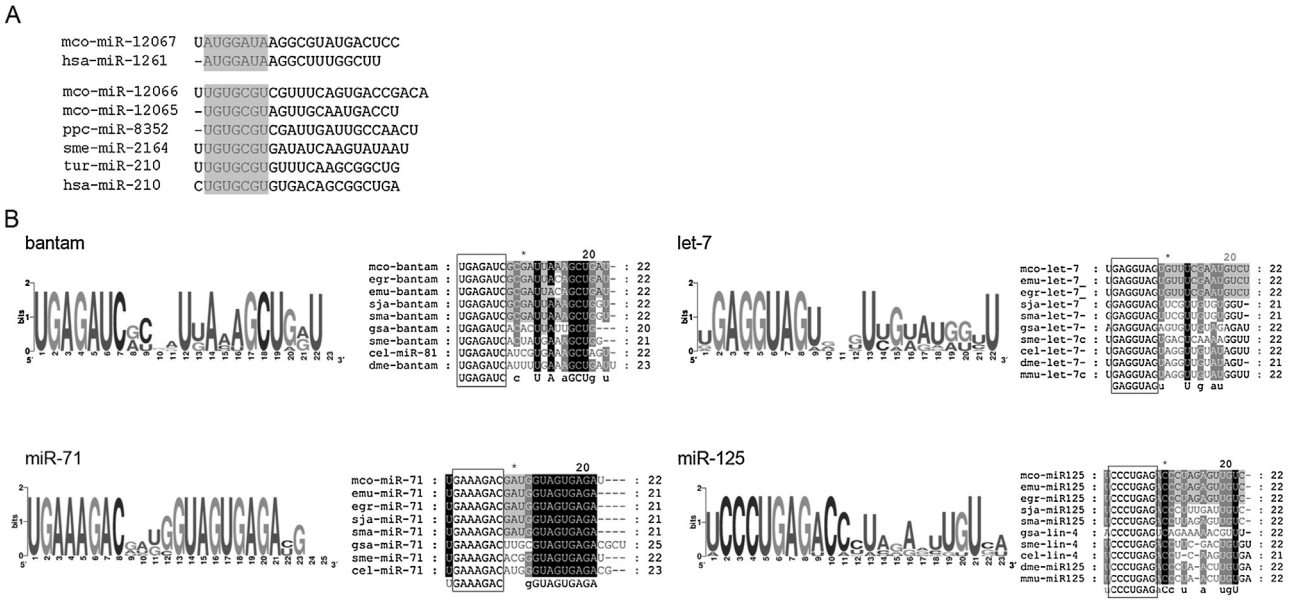

Fig. 7. – Sequence alignments and logos of selected *Mesocestoides corti* miRNAs. A) Seed conservation from two of the new candidates found in *M. corti*: *Homo sapiens (hsa), Pristionchus pacificus (ppc), Tetranychus urticae (tur)*. Alignments were made using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The seed regions are marked with a black rectangle. The identical nucleotides are marked in black and different shades of grey, according to the number of species which share them. B) Sequence logos and alignments of selected *Mesocestoides corti* miRNAs with ortholog miRNAS from *E. multilocularis* (emu), *E. granulosus* (egr), *S. japonicum* (sja), *S. mansoni* (sma), *G. salaris* (gsa), *S. mediterranea* (sme), *C. elegans* (cel), *D. melanogaster* (dme), *M. musculus* (mmu).

gent annotation pipeline used in this work, compared to that used for the close related *E. granulosus* and the trematode *S. japonicum* [27,45]. In line with that, the number of new miRNAs identified in *M. corti* is similarly low as those found in the cestodes *E. canadensis* (5) and *E. multilocularis* (3), and in the trematode *F. hepatica* (5) [25,26,44]. Regarding the comparison with the more distantly related *S. mediterranea* (Turbellaria versus Neodermata), the lower number of new miRNAs found in *M. corti* (6 versus 45) reflects once again lower complexity characteristic of the parasitic way of life.

Despite of the 3p arm processing bias, usual among parasitic platyhelminths, a lack of conservation in arm processing was observed for some *M. corti* miRNAs, particularly those that presented expression from both arms of the hairpin. For instance, the pattern of arm processing in miR-153 and miR-190 was not conserved in most organisms, as mir-190 displays a 5p arm usage pattern in *S. japonicum* and *E. granulosus* [27,51], while miR-153 displays a 3p arm usage pattern in *E. multilocularis* [25]. Interestingly, the pattern of arm expression of *M. corti* miR-153 was also reported in *E. canadensis* and *E. granulosus* [25,27], suggesting that it could be conserved among some cestodes. Another miRNA that did not show conserved arm expression pattern was mir-7b, which displayed a 5p arm usage in *E. canadensis* and *E. granulosus* [26] while

in *M. corti* the 3p arm was the more expressed. The absence of arm usage conservation regarding miR-153, miR-190 and miR-7b in *M. corti* could imply different roles in the biology of these cestodes, since each mature miRNA from the same precursor would target different genes.

We analysed miRNA abundance and found that few miRNAs were expressed at high levels. The highly expressed *M. corti* miRNAs are shared between both stages, and represent ~90% of total miRNA expression. High expression of let-7-5p, miR-10-5p, miR-71-5p and bantam-3p was also observed in other cestodes as *E. multilocularis* and *E. canadensis* [25], and other platyhelminths, like *F. hepatica* adults [52] and *S. japonicum* (in adults and infective larval stages) [45]. In line with that, the mir-10 family, which is highly conserved across metazoans, has been implicated with *Hox* gene regulation, embryonic development, and even cancer [53], and let-7 has been described as a key regulator to ensure the normal development during larval to adult transition both in worms and flies [54]. miR-71, in turn, increases life span via insulin-receptor/phosphatidyl inositol 3-kinase pathway in *C. elegans* [55]. This function may be conserved in *M. corti*, as human insulin is capable of stimulating *M. corti* survival, asexual reproduction, and tyrosine-phosphorylation status in culture [56] and deserves further investigation.

M. corti life cycle involves a number of different life stages and the parasite's development is a complicated and dynamic process [9,57,58], in which miRNA gene regulation could have an important role. We found several miRNAs differentially expressed between the TT and ST stages, which may be functionally involved in regulating stage-specific features. miRNAs up-regulated in the larval stage (TT), could act as repressors of development of the reproductive tissues or controlling reproductive fission, as TT are able to multiply asexually in the intermediate host, being important cell growth and cell division processes for reproduction [58]. We found mi*R*-36 up-regulated in TT. The mir-36 family has been shown to be enriched in planarian neoblasts [59] and is up-regulated in *E. multilocularis* with respect to *E. canadensis* [25], which is in line with the higher regenerative capacity of planarians and the faster proliferation of *E. multilocularis* metacestodes. Therefore, mi*R*-36b in the TT stage of *M* corti, could be regulating proliferation by asexual reproduction. Conversely, miRNAs up-regulated in the ST stage could enhance the development of testes and ovaries or repress asexual reproduction [50]. We found miR-125, up-regulated in the ST stage. In vertebrates, miR-125 regulates somitogenesis through the Notch signaling pathway [60]. The Notch signaling pathway, involved in bilaterian development is present in cestodes [5], so it would be interesting to further investigate the role of this miRNA in *M. corti* development through prediction of assessing miR-125 targets and/or functional studies. On the other hand, antisense-mediated inactivation of miR-2 in *Drosophila* produces embryos with defects in head and posterior abdominal segments [61]. Therefore, miR-125 and miR-2 family in the ST stage of *M. corti*, could be mediating processes like correct strobilum formation (miR-2 via regulation of pro-apoptotic pathways) and signal transduction for sexual maturation (miR-125 via Notch pathways).

Interestingly, miR-190, miR-125, mi*R*-31 were also recently reported as up-regulated in the *E. granulosus* adult stage, while the mir-2 family was more expressed in the pre-adult stage [27], meaning that these miRNAs could be regulating similar processes involved in both *E. granulosus* and *M. corti*. In addition, miRNAs exclusively expressed in *M. corti* TT or ST were identified, suggesting stage-specific roles. Two of these stage-specific miRNAs, miR-7a-5p and miR-12071-5p, showed low expression levels, implying that stage specificity should be confirmed with higher coverage approach.

Recent studies have described that several miRNA families are missing in platyhelminths, especially in the parasitic lineages [30,44]. Our results confirm a reduction of the miRNA complement in *M. corti*. However, we found that the loss of conserved miRNA families in this endoparasitic platyhelminth is smaller than that reported by [30] for *S. japonicum* and *E. granulosus*. This difference in the loss of conserved miRNAs is due to the finding in *M. corti* of mir-92, mir-184, mir-279 and mir-281, along with mir-36 and mir-67, reported as lost in cestodes. Therefore, the proportion of conserved miRNA losses in this cestode parasite seems to be lower than previously reported.

In agreement with this previous studies, a loss of conserved miRNA families among platyhelminths parasites was confirmed in *M. corti*, and could be related to a loss of targets [62] or reduced morphological and metabolic complexity [63], associated with the parasitic way of life. This correlates with the low morphological complexity and gene repertoire reduction, given by the absence of loss of the gut and also the loss of several conserved gene families, for example, homeobox gene families, genes essential for peroxisomes, and fatty acid biosynthesis [5].

A recent study in *E. granulosus* [27] reported mir-96 and mir-184 as absent in this species. In this study we have identified both miRNAs expressed by *M. corti* TT and ST samples. In addition, these authors described mir-92 as exclusive of the free-living flatworm *S. mediterranea*, and absent in Neodermata but we found two members of the mir-92 family expressed in *M. corti*: mi*R*-3479a and mi*R*-3479b. These findings agree with two recent studies in *E. multilocularis, E. canadensis* and *E. granulosus* [25,26]. On the other hand, members of the mir-8 and mir-1992 families were not identified in *M. corti*, although they were reported as present in *Echinococcus* spp [25–27]. Moreover, miR-210 was identified for the first time in platyhelminth parasites and this should be from now on considered in miRNA evolutionary studies.

In this study, three miRNA clusters were reported. However, the number of miRNA clusters may be larger than that, due to the fragmented nature of the assembled draft genome sequence. New clusters may be identified upon completion of the *M. corti* genome sequence assembly. Regarding miRNA cluster conservation, we confirmed that mir-71/2 cluster is widely conserved among protostomes (Ecdysozoan and Lophotrochozoan) as previously described [64], while mir-277/4989 is conserved among cestodes, as it was previously identified in *Echinococcus* spp. and *Hymenolepis microstoma* [24,26,27,65]. There is only one copy of the mir-71/2 cluster in *M. corti*, in contrast with other platyhelminths like *G. salaris*, *S. japonicum*, and *S. mansoni*, where two copies of this cluster have been reported [30]. This might suggest functional shrinking in cestodes as proposed by [65]. On the other hand, while the mir-1/133 cluster is widely conserved across metazoan species [66], its miRNA components seem to be far apart from each other, since the average lengths for platyhelminth miRNA clusters is between 200 bp and 500 bp [67]. However, it has been reported that this particular cluster dramatically varies in length between species, for example in *Echinococcus*, comprises 12 kb [65], and in *D. melanogaster*, the miRNA genes are separated by 50 kb or more [66]. Whether both miR-1 and miR-133 are part of a single transcriptional unit in *M. corti* is an issue that remains to be investigated.

Finally, the divergence of the *M. corti* miRNA sequences found at the nucleotide level with respect to those from other organisms, including other platyhelminths, may likely indicate an accelerated evolution of these miRNAs in the *M. corti* lineage. This could imply specific roles for these miRNAs in development, survival and/or host-parasite interaction. In addition, this may reflect the more complex life cycles of parasitic species and their ability to adapt to different environments. Those miRNAs conserved among cestodes could share functional conservation, and *M. corti* could represent an ideal model for the study of the role of this miRNAs in the biology of this parasites.

In this work, we provide detailed information on the *M. corti* miRNA repertoire, including the first experimental report on the

miRNA expression profiles of the tetrathyridia and strobilated worm. The results reported here show that both life stages display some differentially expressed miRNAs, including some stage specific. In addition, we performed a systematic analysis on the post-transcriptional modifications of each identified miRNA. Conservation of *M. corti* miRNA features, repertoires and expression levels among other cestodes further validate this parasite as a model for the study of biological aspects of cestode parasites and will allow functional studies of miRNAs involved in strobilation process that are difficult to perform in zoonotic parasites such as *Echinococcus* spp. The data obtained from this study will open a way for deep investigation of the parasite biology and host parasite interaction. The findings of differential expression in *M. corti* will allow a deeper understanding more about the biology of development of the parasite.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2016.08.004.

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5. CAPÍTULO 2 – Análise do transcritoma de dois estágios do desenvolvimento do parasito cestódeo *Mesocestoides corti*

Manuscrito a ser submetido para publicação na revista *Gene*

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Transcriptomic profile of two developmental stages of the cestode parasite *Mesocestoides corti.*

Contribuição dos autores:

TB, GO e HB planejaram os experimentos. TB, FA e AS realizaram os experimentos. FP contribuiu com os experimentos. TB, LM, NM, e LK processaram os dados. TB analisou os dados. TB, LK, MR e HB discutiram os resultados. TB e HB escreveram o manuscrito. TB e HB revisaram criticamente o manuscrito.

Transcriptomic profile of two developmental stages of the cestode parasite *Mesocestoides corti*.

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Abstract

Cestode development involves complex morphological and physiological changes. Unfortunately, such interesting developmental phenomena have not received the deserved attention due to difficulties in obtaining and manipulating pathogenic cestodes. This has resulted in a limited knowledge about the molecular signals and repertoire of genes involved in these developmental events. Here we performed a differential expression analysis of genes between two developmental stages of the model cestode *Mesocestoides corti*. A RNA-seq-based approach was used to obtain the transcriptome of the larval (tetrathyridium) and strobilated worm stages. We found transcriptional evidence for 19,053 transcripts, including isoforms and previously not annotated genes. 66 tetrathyridium and 136 strobilated worm-specific transcripts were found, as well as differentially expressed genes (342 and 559, respectively), suggesting regulation of stage-specific features. Differential expression and GO term enrichment analysis showed upregulated in both stages, genes associated with cytoskeleton, metabolism and oxidation-reduction processes. Genes and processes enriched in the tetrathyridium reflect the events this larval stage undergoes in the intermediate host, such as asexual reproduction and budding processes, as well as active migration from the peritoneum to the liver and vice versa. Development, cell growth and morphogenesis are processes that seem to be enriched in the strobilated worm, including the upregulation of numerous transcription factors, protein kinases and histones. This work significantly contributes to the knowledge of the parasite expression profile by increasing the number of sequenced transcripts and through functional annotation of several

genes.

Keywords – *RNA-seq, DEG, trascriptome, strobilation, segmentation, Cestoda, Platyhelminthes.*

1. Introduction

Cestode tapeworms are etiological agents of many diseases such as cystic echinococcosis and neurocisticercosis caused by the *Echinococcus* and *Taenia* genera, respectively, two of the main neglected tropical diseases recently considered by the World Health Organization (WHO, 2016). Despite the fact that these disease-causing cestode species have been extensively studied, some aspects that could also be relevant for cestodiasis control, such as cestode development, have been left aside (Ito, 2015).

Cestode development involves complex morphological and physiological changes that may or may not be associated with changes between host species (Brehm et al., 2006; Olson, 2008; Olson et al., 2012). For instances, larval forms of eucestodes (“true” tapeworms) undergo strobilation to generate preadult or adult forms with increased reproductive capability (Olson et al., 2001). Strobilation includes proglottization (generation of serially repeated reproductive organs) and, in most eucestode species, includes total or partial segmentation (Olson, 2008). Unfortunately, such interesting developmental phenomena have not received the deserved attention due to difficulties in obtaining and manipulating pathogenic cestodes. One obstacle, for instance, is the *in vitro* culture of these parasites, where the most advanced culture system are the metacestode forms of *Echinococcus multilocularis*, limited by the requirement of co-culture with host cells or host-derived factors (Vendelova et al., 2016b). Even more, there is not a suitable system for the development of the larval form to the adult parasite, and when achieved there are high variations between *in vitro* generated material and *ex vivo* harvested material, as recently determined by

Dezaki et al. (2016) for *Echinococcus granulosus.* This has resulted in a limited knowledge about the molecular signals and repertoire of genes involved in these developmental events

Cestode orthologs of some developmentally related genes have been identified in the past few years (reviewed in Brehm, 2010 and Olson et al., 2012). However, the set of identified cestode developmental genes or proteins is still relatively small and insuficient to define whole developmental pathways and associate them with typical cestode developmental processes (García-Montoya et al., 2016; Huang et al., 2016), including proglottization or body segmentation. Therefore, further investigation to identify other developmental genes is important for a more comprehensive understanding of cestode developmental pathways and for functional studies, including response to host-derived stimuli (Olson et al., 2012).

Cestode gene discovery and expression data has benefitted greatly from transcriptomic surveys and genome sequencing efforts, including data from *Taenia solium* (Aguilar-Díaz et al., 2006; Tsai et al., 2013), *E. multilocularis* (Tsai et al., 2013), *E. granulosus* (Parkinson et al., 2012; Tsai et al., 2013; Zheng et al., 2013), *Hymenolepis microstoma* (Tsai et al., 2013) and, more recently, *Taenia saginata* and *Taenia asiatica* (Wang et al., 2016). This genetic information has been applied to understanding a number of metabolic mechanisms used for parasite growth and during host-parasite interactions.

Mesocestoides corti is a well-established model organism for studying the complex life cycle of cestodes and the mechanism underlying development and host-parasite interactions (Bizarro et al., 2005; Laschuk et al., 2011; Markoski et al., 2003). The draft version of *M. corti* genome sequence has recently become

available, but transcriptomic data of this cestode parasite is still unavailable. Recently the miRNA repertoire of larvae and strobilated worm was characterized by our group (Basika et al., 2016). Now with the aim of finding genes involved in the developmental process of this parasite we report the transcriptomic profile of those two developmental stages. In this work the transcriptome of *M. corti* was sequenced using tissues from whole worms at two key developmental states – larvae (tetrathyridia) and strobilated worm – to provide a resource for studying the development to adult form and more specifically the strobila development (strobilation process) in this parasite.

2. Materials and methods

2.1. Parasite material

Mesocestoides corti larvae (tetrathyridia, TT) were maintained by alternate, serial passages in Wistar female rats and BALB/c female mice as previously described (Markoski et al., 2003). Experimental hosts were infected by intraperitoneal inoculation and, after 3 months, larvae were collected and used for experiments. Only TT from up to the third serial passage in mice were used for the experiments. Biological triplicates were used; each replicate produced using TT obtained from a single mouse host.

Larvae freshly collected from mice were washed six times in PBS plus streptomycin-penicillin (5 U/ml) and used immediately for culture. TT (n = 125) were kept in culture for 24 h in modified RPMI 1640 medium with 10 % FBS (McRPMI; Markoski et al., 2003) at 37°C and 5% CO_2. Then, larvae were washed three times with PBS, homogenized in Trizol reagent (Life Technologies) and

stored at -80°C until use for RNA extraction.

Strobilated worms (ST) were obtained from TT (n = 60) cultured under strobilation-inducing conditions as previously described (Markoski et al., 2006). Briefly, TT (n = 60) were incubated with trypsin 0.662 % (w/v) in McRPMI for 24 h at 39°C and 5 % CO_2 for strobilation induction. Induced TT were then cultured in McRPMI without trypsin at 39°C and 5 % CO_2 until complete strobilation, which took around 10-12 days. The culture medium was replaced by fresh McRPMI every 48-72 h and the worms were inspected daily under a Zeiss inverted microscope to follow TT strobilation process and, eventually, to assess the percentage of those that reached the ST stage (which undergone both proglottization and segmentation). Only worm batches with a strobilation rate >90% were used for experiments. ST were then washed three times with PBS, homogenized in Trizol reagent and stored at -80°C until used for RNA extraction.

All experimental procedures for *in vivo* maintenance of M. corti TT in mouse and rat hosts were previously approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul (Project no. 25726).

2.2. RNA extraction

RNA samples were obtained from independent triplicate *in vitro* cultures of TT and ST. Total RNA was extracted using Trizol reagent, according to manufacturer's instructions. After extraction, RNA samples were treated with DNAse I (Sigma) to remove any DNA genomic contamination. RNA concentration and integrity was assessed using Nanodrop 2000 (Thermo) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

2.3. cDNA library construction and sequencing

For cDNA library construction, 10 µg of total RNA were used as start material. For each developmental stage, TT and ST, three libraries were constructed from independent biological replicates. Libraries were constructed using the Truseq RNA Sample Prep Kit V2 (Illumina) according to manufacturer's instructions. The average fragment size in the libraries was 300 bp. rRNA was depleted using RiboMinus Eukaryote kit for RNA-seq (Invitrogen) and following manufacturer's instructions. The resulting RNA was fragmented, purified and cDNA synthesis was carried out using TruSeq RNA sample preparation kit (Illumina), accordingly to manufacturer's instructions. Samples were then diluted in a Tris-HCl 0.1 % Tween solution, loaded in a flow cell and subjected to 600 sequencing cycles (2 x 300 bp) using MiSeq Reagent Kit v3 in a MiSeq Sequencing System (Illumina).

2.4. Source of genome assemblies and annotation

The draft genome sequence of *M. corti* (version 1.0.4) and CDS annotation were retrieved from WormBase ParaSite database (http://parasite.wormbase.org/, Helminth Genomes Consortium). rRNA sequences from *M. corti* and other flatworms (Mallatt et al., 2012) were downloaded from NCBI (http://www.ncbi.nlm.nih.gov). All rRNA sequences were used to build an in-house database for bioinformatics rRNA depletion of sequenced reads.

2.5. In silico *analyses of* M. corti *transcripts*

Raw reads quality was assessed using the FastQC tool (version 0.11.4; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). These raw data

were pre-processed using Trimmomatic (version 0.36; Bolger et al., 2014) for adaptor and low quality reads (phred score >30) removal. Trimmed reads were initially aligned to the draft of the *M. corti* genome sequence using Bowtie2 (version 2.2.3.; Langmead and Salzberg, 2012). Additionally, Bowtie was used for *in silico* removal of reads mapping to rRNA. Resulting reads where collapsed using the fastx_collapser utility from the FASTX-Toolkit (version 0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/index.html).

Collapsed reads were mapped and the transcripts were assembled using Tuxedo pipeline (Tophat version 2.0.12, Cufflinks version 2.2.1; Trapnell et al., 2012). Individual reads from each sample were aligned to the draft version of the *M. corti* genome sequence with the associated annotation file. Mapping rates for each sample were assessed in the Tophat outputs. Mapped reads were assembled into transcripts and the relative abundance of transcripts for each gene was assessed with Cufflinks. Transcript abundance was expressed in fragments per kilobase of exon per million fragments mapped (FPKM). The transcripts identified by Cufflinks were first processed with Cuffcompare to classify the transcripts based on the existing annotation.

Final transcriptome assembly for each life-cycle stage was performed by Cuffmerge and differential expressed genes were identified by Cuffdiff. Prior to differential expression analysis, the correlation between independent biological replicates from each sample type (TT or ST) was assessed. For this purpose, transcript FPKM values within a replicate were plotted against transcript FPKM values in the other replicate and between TT and ST samples. Correlation coefficients were calculated for all sample comparisons. All transcripts identified in this study were considered for this analysis. In the Cuffdiff output, transcripts

with -1 ≥ foldchange ≥ +1 and FDR (False Discovery Rate) adjusted p-value < 0.05 were considered differentially expressed.

2.6. Functional classification of differentially expressed genes

Functional annotation of differentially expressed genes was performed with the Blast2GO v.3.3 software (Conesa and Gotz, 2008). Annotated sequences were mapped to gene ontology (GO) terms using default settings. Enrichment analysis of GO terms specific to each developmental stage was performed with Cytoscape's BiNGO app (Maere et al., 2005), using the hypergeometric test with the Benjamini and Hochberg correction (FDR) with an 0.01 significance level. The resulting GO lists were summarized using the REVIGO online tool (http://revigo.irb.hr/; Supek et al., 2011).

3. Results

3.1. Summary of RNA-seq data and transcript assembly

With the aim of identifying differentially expressed genes between the *M. corti* larval (TT) and strobilated worm (ST) stages, a RNA-seq-based approach was used. Biological triplicates were sequenced for each life-cycle stage and Table 1 shows the summary of sequencing results. A total of 70.2 million of paired-end reads were obtained among all the sequenced samples. Following sequencing and quality filtering, 54.9 million of paired-end reads (~78 %) remained, and, after rRNA depletion, 27.5 million reads (39 %) were obtained.

Table 1 - Summary of sequence statistics for *M. corti* RNA-seq data

Stage	Replicate	Raw reads	Trimmed reads	Reads after rRNA depletion	Mapped reads	Mapped reads (%)
Tetrathyridia	1	8.998.626	6.972.374	4.357.828	2.602.272	59,7
	2	11.249.538	8.761.550	4.614.184	2.576.546	55,8
	3	19.112.078	14.835.506	7.460.850	3.791.615	50,8
Strobilated worm	1	6.613.144	5.187.492	2.414.372	1.148.349	47,6
	2	8.463.782	6.661.892	3.196.624	1.531.178	47,9
	3	15.835.158	12.476.388	5.495.026	2.399.539	43,7
Total	-	70.272.326	54.895.202	27.538.884	14.049.499	-

After the initial processing stage, around 50% of the clean reads (from 1.15 to 3.8 million in the different samples) were aligned to the draft version of the *M. corti* genome. (Table 1). After Cufflinks assembly, a total of 19,053 transcripts were obtained from the total of 14 million aligned reads. Based on the Cuffcompare output (Table 2), ~45 % of these transcripts were identified as being a complete match to a known *M. corti* genome annotation, while ~24 % were considered novel isoforms and ~24 % were considered potential new transcripts.

3.2. Differential expression analysis

Before differential expression analysis, the variation between replicates and life stage samples was assessed with a scatter plot of FPKM values of all detected transcripts, and the Spearman's correlation coefficient was calculated between the analysed samples (Figure 1). As shown in the scatter plots, the biological replicates show less dispersion and higher correlation coefficients than the samples of the two different life-cycle stages, indicating that there were little variation among the replicates and a clear separation between TT and ST samples.

Differential expression was assessed with Cuffdiff and for all significant results (p-value <0.05), only the samples with $-1 \geq \log_2(\text{fold change}) \geq 1$ were considered for further analysis. The sign of this $\log_2$ (fold change) value was used to partition the differentially expressed genes into upregulated genes for each stage. The total number of differentially expressed genes matching these criteria was 901 (Figure 2), with 342 genes upregulated in the TT stage and 559 genes upregulated in the ST stage. Additionally, genes with FPKM > 0 for one sample and FPKM equal to zero in the other were considered stage specific. With this

criterium, 66 TT-specific genes and 136 ST-specific genes where identified.

Table 2 - number of detected transcripts by annotation classification by Cuffcompare

Class code[a]	transcripts, *n*	%
=	8594	45,11
C	1	0,01
J	4708	24,71
E	1	0,01
I	0	0
O	1021	5,36
P	0	0
R	0	0
U	4571	23,99
X	159	0,83
S	1	0,01
.	0	0
Total	19.053	100

a "=", Complete match of intron chain; "c", contained; "j", potentially novel isoform (fragment): at least one splice junction is shared with a reference transcript; "e", single exon transfrag overlapping a reference exon and at least 10 bp of a reference intron, indicating a possible pre-mRNA fragment; "l", a transfrag falling entirely within a reference intron; "o", generic exonic overlap with a reference transcript; "p", possible polymerase run-on fragment (within 2 Kb of a reference transcript); "r", repeat, determined by looking at the soft-masked reference sequence and applied to transcripts where at least 50% of the bases are lower case; "u", unknown, intergenic transcript; "x", exonic overlap with reference on the opposite strand; "s", an intron of the transfrag overlaps a reference intron on the opposite strand (likely due to read mapping errors), ".", tracking file only, indicates multiple classifications.

Table 3 shows the 15 most upregulated genes for each analysed stage. Among the most upregulated genes in the TT stage genes coding for proteins of the CAP superfamily (10 genes); fatty acid binding protein (FABP); peptide methionine sulfoxide reductase; aldo-keto reductase superfamily member; and, finally, 5'-AMP activated protein kinase were identified. On the other hand, among the most upregulated genes in the ST stage, genes coding for tubulin (2 genes); ADP ribosylation factors (2 genes); tyrosine protein phosphatase receptors (2 genes); pyruvate kinase; glyceraldehyde 3 phosphate dehydrogenase (GAPDH); and, finally, Hsp70 protein (2 genes) were identified.

Genes regarded as stage-specific are shown in Table 4. For TT, genes coding for proteins of the CAP superfamily (14 genes), and CD36 class B scavenger receptor were identified. Among ST-specific genes, genes coding for proteins of the CAP superfamily (2 genes); calcium binding protein; an enzyme involved in protein glycosylation (B3GAT2); and an antioxidant enzyme (PHGPx) were identified. Other genes identified as ST-specific were those coding for an ester hydrolase enzyme; and a protein from the CCR4-NOT complex.

The repertoire of shared but differentially expressed genes (Supplementary File 1 – vide Apêndice 5/CD) included, in both samples, genes coding for proteins related to cytoskeleton (tubulin and dynein), and proteins involved in molecule transport (major facilitator superfamily) and calcium signaling (EF hand domain). Related to metabolism, upregulated genes in both samples were associated to carbohydrate metabolic processes (glycoside hydrolase and glycosyl transferase) and lipid binding proteins (FABPs, antigen B subunit and apolipoprotein). Several genes coding for enzymes related to glycolysis (hexokinase, GAPDH, enolase) were upregulated in the ST stage, proteins possibly required for high metabolic activity during development.

Several genes coding for proteins containing transcription factors domains were expressed in both stages (BTB:POZ domain, LyM domains), however, some genes coding for transcription factors were upregulated only in the ST stage, such as TFIID, FAR-1, Von Willebrand factor and enhancer of yellow 2, suggesting specific roles in the strobilated parasite. Other genes upregulated in both stages were genes coding for proteins involved in protein turnover, mainly proteasome related (ubiquitin ligases, desumoylating isopeptidase 1, ubiquitin conjugation proteins), and oxidation and reduction processes (glutathione S transferase,

thioredoxin domain containing).

Interestingly, TT-exclusively upregulated genes were those coding for annexins; and phospholipid-metabolism related proteins, such as the LicD family and proteins containing a C2 domain. Other abundant genes were coding for

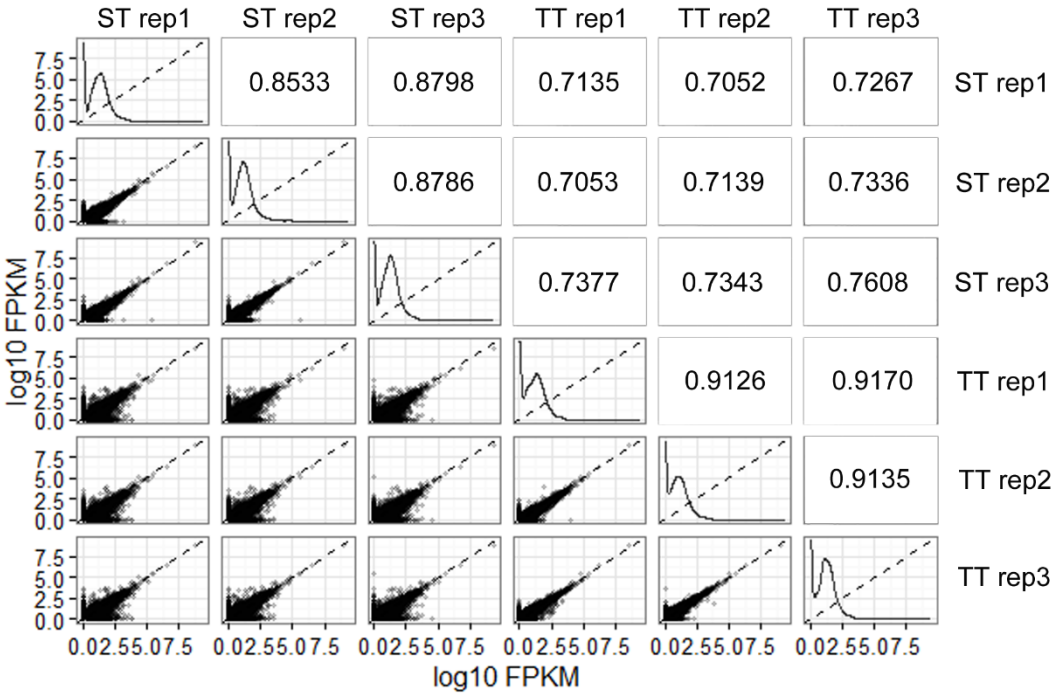

Figure 1 – Correlation analysis of sequenced samples - Scatterplots of the log10 expression values (FPKM) for the detected genes between biological replicates and developmental stages. Spearman's correlation coefficients are indicated in the top half of quadrants.

sodium-neurotransmitter symporters, involved in the release, re-uptake and recycling of neurotransmitters at synapses.

The repertoire of upregulated genes in ST stage exclusively contained genes associated to cell cycle and cell growth, including *cyclin B3*, *regulator of cytokinesis*, *cyclin dependent kinase*, *pumilio*, *m-fase inducer phosphatase* and *wnt11*. Other ST-upregulated genes were coding for proteins involved in DNA replication, such as histone H1-like, H2A and H3; and genes involved in signalization, including genes coding for proteins containing kinase domain, Rho-GTPase-activated protein, protein phosphatase, and fibronectin III

(phosphatidylinositol and tyrosine phosphatase). Domains associated with protein- protein interaction were also abundant, such as WD40 repeat (cell division cycle 20 homolog protein, G protein, SEC13); ankyrin repeats and BTB/Kelch-associated, both mainly associated to ubiquitination and protein turnover. Taken together, genes associated with transcription, protein turnover and signalization were upregulated in the ST stage.

Additional genes found upregulated in the ST stage were those coding for various metalloproteinases (M17 family), and two proteins related to meiosis, radial spoke head 1 and a protein containing an N2227-like domain.

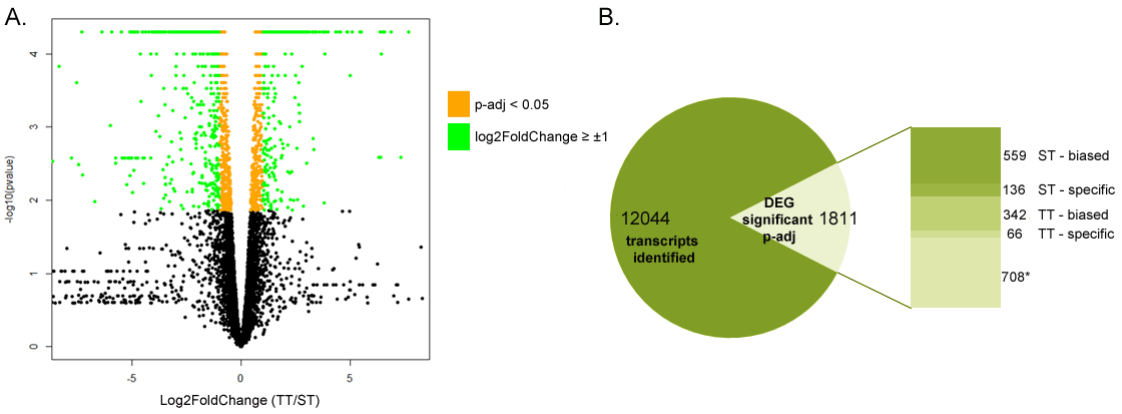

Figure 2 – Differential expression analysis – A. Volcano plot of the log10 (p-adj) versus log2 (Fold Change) for TT-ST comparison. Genes with p-adjusted < 0.05 are displayed in orange, while genes within this group and with a Log2 fold change ≥ ±1 are displayed in green. B. Pie chart with numbers of differentially expressed and stage-specific genes identified. * marks genes that match the p-adj criteria but not the Log2 fold change criteria.

3.3. GO term enrichment analysis

The complete functional enrichment analysis for TT and ST transcriptomes (Supplementary File 2 – vide Apêndice 6/CD) assigned 649 different GO terms to the two life stage samples, 208 to TT samples and 490 to ST samples. Comparisons of the assigned GO terms between the two stages revealed only

49 GO terms shared between TT and ST (Figure 3), with 159 GO terms found only for the TT stage and 441 GO terms found only for the ST stage. This clearly shows a more simple functional profile in the TT stage compared to ST, possibly indicating more biological complexity in the latter.

Functional profiles based on GO categories were also different between the two stages. In TT, the top 40 GO terms (Figure 3, Supplementary File 2 – vide Apêndice 6/CD) were represented predominantly in the biological process (BP) category by terms for negative regulation of development, carbohydrate transport, ion transmembrane transport, ion homeostasis and localization. In the cellular component (CC) category were represented by the subcategories extracellular, vesicle and membrane; and, in the molecular function (MF) category by transmembrane transport and Ca^{2+} dependent phospholipid binding. While in ST, the top 40 GO terms were represented in BP by drug transport, metabolic process and phosphorylation; in CC by cilium, cell projection and extracellular subcategories; and in MF by catalytic activity and drug transport.

To better summarise the functional information of significant GO terms, the REVIGO online tool was used to analyse TT and ST functional profiles (Figure 4, Supplementary File 3 – vide Apêndice 7/CD, and Supplementary File 4 – vide Apêndice 8). As observed for the top 40 enriched GO term in each stage, functional profiles were different for each of the stages analysed and more

Table 3 - Top 15 up-regulated genes identified in *M. corti* tetrathyridia (TT) and strobilated worm (ST)

TT-biased

no	gene_id	Locus	FPKM ST	FPKM TT	log2(FC)[a]	Description (blastn)	ID InterPro	Interpro Description	Length
1	XLOC_011999	MCOS_scaffold0001562:918-1855	11,8371	2435,44	7,68	---NA---	IPR014044	CAP domain	869
2	MCOS_0000939901	MCOS_scaffold0000533:19576-21139	2,65635	429,386	7,34	---NA---	IPR014044	CAP domain	639
3	XLOC_002859	MCOS_contig0000459:31617-32368	41,8231	4974,36	6,89	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	485
4	XLOC_010719	MCOS_scaffold0000553:7016-13685	29,047	2759,13	6,57	---NA---	IPR001283	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	946
5	XLOC_008887	MCOS_scaffold0000241:21798-23533	2,11409	183,415	6,44	---NA---	IPR014044	CAP domain	1084
6	XLOC_007039	MCOS_scaffold0000066:69223-71029	4,66775	387,196	6,37	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	939
7	XLOC_005885	MCOS_scaffold0000007:186690-241057	7,18704	244,421	5,09	GLIPR1 protein 1 [*Echinococcus granulosus*]	IPR014044	CAP domain	1003
8	MCOS_0000263001	MCOS_contig0000457:5910-7818	69,3592	2052,82	4,89	fatty acid binding protein b [*Mesocestoides vogae*]	IPR000463	Cytosolic fatty-acid binding	435
9	XLOC_008584	MCOS_scaffold0000206:81504-83264	6,73835	153,096	4,51	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	906
10	XLOC_003153	MCOS_contig0000548:22595-24918	11,0115	225,323	4,35	GLIPR1-like protein [*Echinococcus granulosus*]	IPR014044	CAP domain	1409
11	MCOS_0000307001	MCOS_contig0000616:3713-6425	11,265	180,299	4,00	peptide methionine sulfoxide reductase [*Hymenolepis microstoma*]	IPR002569	Peptide methionine sulphoxide reductase MsrA	570
12	XLOC_006358	MCOS_scaffold0000026:8333-19977	6,07804	89,7871	3,88	hypothetical protein EgrG_000604800	NA	---NA---	715
13	XLOC_002647	MCOS_contig0000402:16069-17620	2,67102	38,3497	3,84	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	804
14	MCOS_0000038301	MCOS_contig0000031:81854-93782	10,2937	144,59	3,81	5'-AMP-activated protein kinase subunit gamma-2 [*Echinococcus granulosus*]	IPR000644	CBS domain	1362
15	XLOC_000671	MCOS_contig0000056:23598-33785	10,6293	148,196	3,80	aldo keto reductase [*Echinococcus granulosus*]	IPR020471	Aldo/keto reductase	751

ST-biased

no	gene_id	Locus	FPKM ST	FPKM TT	log2(FC)[a]	Description (blastn)	ID InterPro	Interpro Description	Length
1	MCOS_0000561501	MCOS_scaffold0000024:149301-155244	198,456	0,503317	-8,62	Tubulin alpha-1C chain [*Echinococcus granulosus*]	IPR000217	Tubulin	2679
2	MCOS_0000198101	MCOS_contig0000291:33371-44160	279,389	0,881388	-8,31	receptor type tyrosine protein phosphatase [*Echinococcus granulosus*]	IPR003961	Fibronectin type III	2193
3	XLOC_006213	MCOS_scaffold0000020:11499-23788	159,906	0,746091	-7,74	phosphatidylinositol phosphatase PTPRQ-like	IPR003961	Fibronectin type III	2842
4	MCOS_0000696501	MCOS_scaffold0000132:65332-71455	704,343	3,8542	-7,51	hypothetical protein EGR_09402 [*Echinococcus granulosus*]	NA	---NA---	129
5	MCOS_0000643101	MCOS_scaffold0000082:60177-65358	98,2269	0,574782	-7,42	pyruvate kinase [*Echinococcus granulosus*]	IPR001697	Pyruvate kinase	1677
6	MCOS_0000562101	MCOS_scaffold0000024:234810-243450	309,481	1,99399	-7,28	tubulin alpha 3 chain [*Echinococcus multilocularis*]	IPR000217	Tubulin	2026
7	MCOS_0000493201	MCOS_contig0002758:74-1712	217,818	1,43653	-7,24	ADP-ribosylation factor family [*Populus trichocarpa*]	IPR005225	Small GTP-binding protein domain	633

8	MCOS_0000642901	MCOS_scaffold0000082:42583-49103	202,102	1,96192	-6,69	tegumental antigen [*Hymenolepis microstoma*]	IPR001372	Dynein light chain, type 1/2	882
9	MCOS_0000829901	MCOS_scaffold0000309:16456-35620	250,71	3,96782	-5,98	---NA---	IPR000941	Enolase	1104
10	MCOS_0000235101	MCOS_contig0000374:31328-32345	330,532	5,48997	-5,91	glyceraldehyde 3-phosphate dehydrogenase [*Schistosoma japonicum*]	IPR006424	Glyceraldehyde-3-phosphate dehydrogenase, typeI	1017
11	MCOS_0000495501	MCOS_contig0002850:28-1586	785,765	13,1285	-5,90	ADP-ribosylation factor A1F isoform 2 [*Theobroma cacao*]	IPR005225	Small GTP-binding protein domain	660
12	XLOC_005697	MCOS_contig0005043:0-519	192,265	3,62332	-5,73	heat shock 70 kDa protein [*Echinococcus granulosus*]	IPR013126	Heat shock protein 70 family	519
13	MCOS_0000174601	MCOS_contig0000239:22329-25993	82,1834	1,54944	-5,73	solute carrier family 2 [*Schistosoma japonicum*]	IPR003663	Sugar/inositol transporter	1329
14	MCOS_0001018801	MCOS_scaffold0000872:8672-10835	90,6475	2,06174	-5,46	---NA---	IPR013126	Heat shock protein 70 family	1638
15	MCOS_0000351201	MCOS_contig0000826:9081-10031	81,2749	1,91923	-5,40	Aquaporin-9 [*Echinococcus granulosus*]	IPR000425	Major intrinsic protein	876

complex, presenting a higher number of semantic clusters, in ST compared to TT.

In TT samples, the summary of GO subcategories enriched for BP were ion transmembrane transport, negative regulation of developmental growth, localization, proteoglycan metabolism, lipid catabolic process, endomembrane system organization and RNA secondary structure binding. Genes upregulated in the TT stage were predominant in 4 CC subcategories, namely integral components of membrane, membrane, extracellular region and extracellular exosome. Regarding MF, genes upregulated in the TT stage seemed to be predominantly involved in transporter activity, calcium dependent phospholipid binding, oxidoreductase activity, and kinase inhibitor activity.

In the ST samples, the summary of GO subcategories enriched for BP were metabolic process, nucleotide phosphorylation, cell morphogenesis, and regulation of catalytic activity. There were also some low abundant categories that deserve consideration, such as microtubule based, cell division and cell growth. Genes upregulated in ST samples were predominant in the CC subcategories integral component of membrane, membrane, cell projection and cytoplasm. Regarding MF category, genes upregulated in ST samples showed to be predominantly involved in hydrolase activity, adenylate kinase activity and macromolecular complex. Other less abundant categories that deserve consideration are glucose binding activity, poly(A) RNA binding, and chromatin binding activity.

Table 4 - Top 15 genes with stage-specific expression identified in *M. corti* tetrathyridia (TT) and strobilated (ST) worm

TT-specific

no	gene_id	Locus	FPKM TT[a]	Description (blastn)	ID InterPro	Interpro Description	Length
1	XLOC_004160	MCOS_contig0001049:9180-12025	324,657	CD36 class B scavenger receptor [*Echinococcus granulosus*]	IPR002159	CD36 family	931
2	XLOC_003760	MCOS_contig0000803:5494-6984	310,749	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	863
3	XLOC_004699	MCOS_contig0001519:236-1444	229,237	GLIPR1-like protein [*Echinococcus granulosus*]	IPR014044	CAP domain	640
4	XLOC_011942	MCOS_scaffold0001376:1192-2732	208,095	---NA---	IPR014044	CAP domain	799
5	XLOC_008992	MCOS_scaffold0000253:35028-36482	177,652	---NA---	IPR001283	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	801
6	MCOS_0000649801	MCOS_scaffold0000087:4998-6468	150,32	GLIPR1 protein 1 [*Echinococcus granulosus*]	IPR002413	Ves allergen	489
7	XLOC_005886	MCOS_scaffold0000007:263442-264898	132,787	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	NA	---NA---	865
8	XLOC_007648	MCOS_scaffold0000114:1-465	130,079	GLIPR1-like protein [*Echinococcus granulosus*]	IPR014044	CAP domain	318
9	XLOC_005567	MCOS_contig0003502:193-949	110,593	peptidase inhibitor 16 precursor [*Rattus norvegicus*]	IPR014044	CAP domain	577
10	XLOC_005677	MCOS_contig0004814:0-551	104,558	Putative protein C6orf89 like protein [*Chelonia mydas*]	IPR014044	CAP domain	370
11	MCOS_0000888001	MCOS_scaffold0000410:4136-5887	100,479	---NA---	IPR001283	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	489
12	XLOC_009618	MCOS_scaffold0000344:33499-35049	95,5858	---NA---	IPR014044	CAP domain	751
13	XLOC_005263	MCOS_contig0002476:1155-2167	90,2948	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	IPR014044	CAP domain	448
14	XLOC_005699	MCOS_contig0005068:94-390	83,4326	cysteine-rich secreted protein 2 precursor [*Mesocestoides vogae*]	NA	---NA---	296
15	XLOC_010282	MCOS_scaffold0000458:17987-19627	82,2911	---NA---	IPR001283	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	905

ST-specific

no	gene_id	Locus	FPKM ST	Description (blastn)	ID InterPro	Interpro Description	Length
1	MCOS_0000200401	MCOS_contig0000297:4471-6018	882,888	Glioma pathogenesis-related protein [*Echinococcus granulosus*]	IPR001283	Cysteine-rich secretory protein, allergen V5/Tpx-1-related	378
2	MCOS_0000488201	MCOS_contig0002575:776-1029	712,116	Calcium-binding [*Echinococcus granulosus*]	IPR002048	EF-hand domain	210
3	MCOS_0000137301	MCOS_contig0000168:267-837	623,185	expressed conserved	NA	---NA---	570
4	MCOS_0000561901	MCOS_scaffold0000024:223149-224496	356,708	Tubulin alpha-1C chain [*Echinococcus granulosus*]	IPR000217	Tubulin	972
5	MCOS_0000285401	MCOS_contig0000529:8699-10414	189,355	Hexokinase [*Echinococcus granulosus*]	IPR001312	Hexokinase	1317
6	XLOC_005121	MCOS_contig0002143:441-3094	144,913	hypothetical protein EgrG_000663300	NA	---NA---	562
7	MCOS_0000380201	MCOS_contig0001011:1431-2800	126,981	B3GAT2 [*Echinococcus granulosus*]	IPR005027	Glycosyl transferase, family 43	801

8	MCOS_0000268901	MCOS_contig0000475:11610-12566	106,106	PHGPx isoform 3 [*Clonorchis sinensis*]	IPR000889	Glutathione peroxidase	456
9	MCOS_0000275801	MCOS_contig0000497:6536-7967	104,587	cysteine rich secretory protein LCCL [*Echinococcus multilocularis*]	IPR014044	CAP domain	492
10	MCOS_0000082501	MCOS_contig0000082:49089-49254	99,2182	nucleolar c7c	IPR018392	LysM domain	165
11	MCOS_0000056001	MCOS_contig0000051:38505-41017	92,3048	Ester hydrolase C11orf54 protein [*Echinococcus granulosus*]	IPR015021	Domain of unknown function DUF1907	797
12	MCOS_0000050001	MCOS_contig0000044:28071-29088	85,9758	FAM78A [*Schistosoma haematobium*]	IPR029638	Protein FAM78	1017
13	MCOS_0000600001	MCOS_scaffold0000048:181511-182189	72,2696	Cnot1 Mif4g Domain - Ddx6 Complex	IPR032191	CCR4-NOT transcription complex subunit 1, CAF1-binding domain	678
14	MCOS_0000360301	MCOS_contig0000875:7373-8228	69,9424	hypothetical protein EgrG_000146000 [*Echinococcus granulosus*]	NA	---NA---	855
15	MCOS_0000513401	MCOS_scaffold0000002:278372-278587	69,2169	Rhabdoid tumor deletion region 1 [*S. haematobium*]	IPR011989	Armadillo-like helical	215

4. Discussion

To get insights on the mechanism underlying *M. corti* strobilation, the transcriptomes of the intermediate larval (TT) stage and of the fully strobilated adult (ST) stage were sequenced. Although there was some contamination with rRNA sequences, over 50% of the sequence reads mapped to the draft version of *M.corti* genome sequence, in a rate similar to that recently reported for *Taenia crassiceps* cysticercus stage transcriptome (García-Montoya et al., 2016).

Since around 25% of the *M corti* genes identified in this study were genes not previously annotated in the draft version of the *M. corti* genome sequence, this work significantly contributes to the knowledge of the parasite expression profile by increasing the number of sequenced transcripts and through functional annotation of several genes. In addition, the transcription of several genes annotated as “hypothetical expressed” was confirmed. Thus, the present report is complementary to genome sequence and may be helpful on the parasite genome assembly and annotation.

Regarding differential expression between samples, a higher amount of upregulated genes and the associated GO term subcategories were identified in the ST stage, possibly indicating that this stage presents a more complex morphology, as well increased metabolic activity, associated with a more differentiated state compared to TT and the fact that the ST stage undergoes sexual maturation and reproduction. In a recent comparative transcriptomic study in *E. multilocularis* there were more upregulated genes in the metacestode stage than the oncosphere (Huang et al., 2016), also reflected by the increased morphological complexity of the metacestode compared to the oncosphere.

Among the genes detected from both TT and ST samples, there were components of the cytoskeleton, in particular a high abundance of genes coding for dyneins, proteins involved in intracellular motility of vesicles and organelles along microtubules. In fact, the dynein light chain domain is expanded in both trematode and cestode parasites (Parkinson et al., 2012). Previous analyses of the cestodes *T. solium*, *T. crassiceps* and *E. granulosus*, have shown that cystoskeletal proteins are among the most expressed (Almeida et al., 2009; García-Montoya et al., 2016; Tsai et al., 2013). This observation might correlate with the plasticity required for the parasite continuous development, size changes, motility, vesicular trafficking and adaptation to the host environment (Maule and Marks, 2006). Our results are in line with such reports, suggesting an important diversification of the protein repertoire dedicated to cystoskeletal dynamics, important in *M. corti* TT, where the parasite larvae have to reproduce asexually by fission and actively migrates through the host, and equally important in ST, where cystoskeletal dynamics are associated with morphological changes, cell growth and division.

A large number of identified genes in the two samples analysed were functionally related to metabolism, including those coding for many well-known metabolic enzymes, such as the glycolytic enzyme pyruvate kinase. Other genes related to glycolysis were only upregulated in ST stage, as those coding for enolase, hexokinase and GAPDH proteins, suggesting that a high metabolic activity is required in this developmental stage. In addition, these enzymes are often reported as secreted in other cestodes such as *E. granulosus* (Pan et al., 2014), and several alternative (moonlighting) functions have been reported for the secreted forms of this enzymes (Lorenzatto et al., 2012; Zhang et al., 2015).

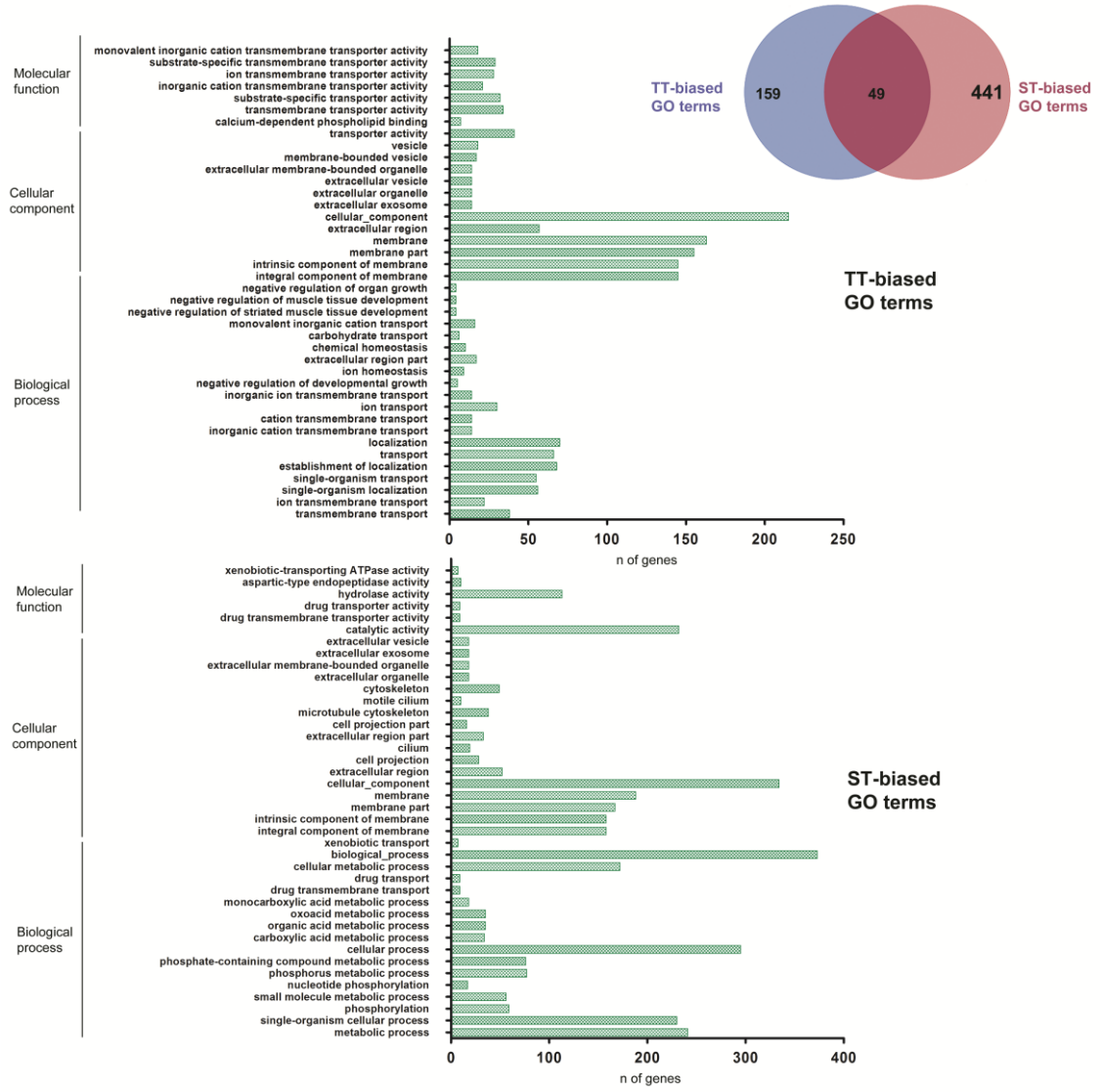

Figure 3 – Gene ontology (GO) term enrichment analysis – Venn diagram showing the number or enriched GO terms in each stage. Barr plots show the number of genes represented in the top 40 enriched GO terms identified in each stage.

These proteins could play roles that may be relevant to parasite growth, development and adaptation to the host environment.

We also found several glycoside hydrolases and glycosyl transferases, which were upregulated in the two life-cycle stages. Proteomic analysis of the ES products from larval stage of *E. multilocularis* (Wang et al., 2015) also revealed high abundance of these proteins. This could suggest that the degradation of complex carbohydrates may form an essential part of the energy metabolism of

both *M. corti* and *E. multilocularis*, once they establish in the intestine of the definitive host or tissues of the intermediate host. In addition, glycosyl transferases and glycoside hydrolases may be involved in tegument remodelling dynamics, in forming the glycocalyx layer on the tegumental surface (Lumsden, 1974).

Cestodes lack the ability to synthesize fatty acids and cholesterol de novo (Tsai et al., 2013). Instead, they take essential lipids from the host using fatty acid transporters and lipid elongation enzymes, as well as several tapeworm-specific gene families, such as *FABP* and the *apolipoprotein antigen B*. Uptake of fatty acid seems to be crucial in *M. corti* TT and ST, in which both *FABP* and *antigen B* genes were found upregulated. Additionally, cestodes have reduced ability to synthesize amino acids (Zheng et al., 2013). Several proteolytic enzymes as well as several amino acid transporters were upregulated in both stages. Therefore, host proteins could be degraded and amino acids then transported inside the parasite cells for protein synthesis.

Oxidative and other types of stress are inherent to the host environment to which a parasite is exposed. Therefore, parasites must cope with oxidants and reactive oxygen species (ROS) derived not only form their own aerobic metabolism, but also from host activated cells such as phagocytes (Parkinson et al., 2012). Several redox-based antioxidant enzymes were present in both TT and ST, and many of them were highly upregulated, suggesting detoxification mechanism against host immune systems in both stages. In line with this, antioxidant enzymes were detected in a proteomic characterization of the excretion-secretion products of *M. corti* TT, possibly involved in the inhibition of inflammatory response (Vendelova et al., 2016a). Other genes upregulated in

both stages were those coding for universal stress proteins (USP), and cytoplasmic proteins associated with stress responses. However, genes coding for Hsp70 were only upregulated in ST. Hsp70 proteins have important roles in protein folding and in protecting cells from stress, and an expansion of this family has been reported for *E. granulosus* and *E. multilocularis* (Tsai et al., 2013), as well as differential expression among adult and metacestodes of *E. granulosus* (Zheng et al., 2013). Both Hsp70 and USPs may be involved in stress response, but expression of Hsp70 in the ST stage may be specially associated to the stressful conditions of strobilation induction, which involves an increase in protein synthesis and degradation (Laschuk et al., 2011).

Among the genes identified exclusively or more abundantly in the TT, genes coding for Venom Allergen-Like protein family as well as other proteins that belongs to the cysteine-rich secretory protein family (CAP domain) were found. Members from this family have been identified in several cestode species, including *M. corti* (McCrisp2; Britos et al., 2007), and were suggested as potential modulators of host immune function and components of sexual development during the infection processes (Silvarrey et al., 2016; Wang et al., 2015). In *M. corti*, McCrisp2 expression was localized mainly at the larvae apical region of tetrathyridia and in the proglottids of segmented worms. Here in this work, several CAP domain containing genes coded for inhibitors, associated with GO terms ‘negative regulation of developmental growth’ were identified in the TT. This suggest repression towards development or sexual reproduction, in a parasite stage where asexually reproduction occurs. Despite this, several CAP were also found upregulated in ST samples, so we cannot rule out equal importance of these proteins in both stages. Regarding other developmental related molecules,

wnt genes were identified upregulated in both stages. *Wnt4* was upregulated in TT samples, while *wnt11* was upregulated in ST samples. In *T. solium wnt4* may take part in the process of metacestode evagination and play a role in scolex/bladder development (Hou et al., 2014), therefore could be also regulating scolex development in the TT stage, when asexual reproduction ocurrs. On the other hand, *wnt11* was found upregulated in response to incubation at male producing temperature in the American alligator (Yatsu et al., 2016), and could maybe regulating male reproductive apparatus development in the strobila of *M. corti* ST. In both cases, these genes seem to be playing roles associated to specific developmental changes of each stage.

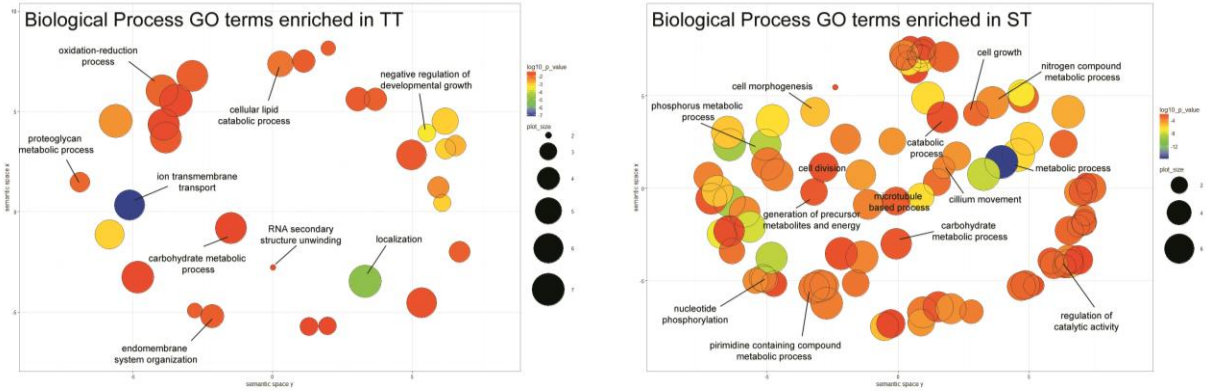

Figure 4 – Summary of enriched GO terms for Biological Process – Scatter plot of the GO terms for biological process summarised by employing multidimensional scaling to the matrix of semantic similarities. Colour is proportional to the P-value obtained in the GO term enrichment analysis. Size is proportional to the term frequency in the GO annotation database, also indicating general terms. Names present the most significant terms in each group of semantically similar terms.

Several genes coding for annexins were upregulated in the TT stage. Annexins can be associated with cellular membranes and they can play roles in vesicular transport. Furthermore, annexins can bind to certain membrane phospholipids in a Ca^{2+}-dependent manner, providing a link between Ca^{2+} signalling and membrane functions. Several genes coding for EF hand domain

containing proteins were identified, therefore enabling annexins to participate in several unrelated events that range from membrane dynamics to cell differentiation and migration (Gerke et al., 2005). It is possible that these proteins are playing essential roles during the budding of TT and, consequently, aiding in the asexual reproduction process.

Overall, genes and processes enriched in the TT stage show this larva as an active stage, rather than a quiescent one. In fact, TT is highly active in the intermediate host, going through asexual reproduction and budding processes, as well as active migration from the peritoneum to the liver and vice versa, and encystation in the hepatic tissue (Hrckova et al., 2010). This active stage status is reflected by the upregulation of certain genes identified in this study, such as proteins involved in cytoskeleton and endomembrane transport dynamics, active catabolic processes (lipid and carbohydrate catabolism) and even in developmental regulation. In addition, this larvae shows a flexible host specificity, being able to infect reptiles, birds and mammals (Smyth, 1987). It would be interesting to further characterize the expressed gene repertoire of TT, in order to identify genes associated with *M. corti* larval flexible host range.

Development, cell growth and morphogenesis are processes that seem to be enriched in ST. Among genes upregulated in this stage there were those coding for a number of growth factors (EGF-like and Von Willebrand growth factors). Those types of growth factors are known to participate in cell growth/division in response to stimuli (Yusuf et al., 2012). Additionally, there were multiple upregulated genes coding for proteins involved in cell signalling (kinase, ATPase, and GTPase domains). A central pathway involved in eukaryotic development, cell proliferation, apoptosis, and embryogenesis is the MAPK/ERK

pathway where extracellular receptor kinases such as EGF-receptors bind their ligands and start a signalling cascade via different intracellular factors leading in gene transcription (Spiliotis et al., 2010; van der Geer et al., 1994). In this work a number of genes coding for proteins involved in this pathway were identified as ST-biased, meaning that cell proliferation and maybe embryogenesis are processes active in this parasite stage. Other protein-coding gene related to proliferation is *pumilio*, hypothesised to have a conserved primordial function in the maintenance of proliferation in stem cells through post-transcriptional regulation, mediated by the binding of the encoded protein of specific sequences in the 3′-UTR of target genes (Wickens et al., 2002). This transcript was previously identified as expressed in *M.corti* by Koziol et al. (2008), and therefore could be mediating active proliferation in the ST stage. Finally, two genes related to meiosis were also identified, suggesting that gametogenesis is occurring in the ST stage.

In summary, in this work is reported the first high-throughput transcriptome analysis of two developmental stages of *M. corti*. Both TT and ST present the upregulation of genes coding for lipid-binding proteins and amino acid transporters to compensate for the loss of genes associated to the synthesis of these molecules, and abundance of genes coding for cystoskeleton and antioxidant proteins. More importantly, this work also provides information about differentialy expressed genes in these two key developmental stages, providing a model for the study of segmentation-strobilation. Finally, this being the first high-throughput transcriptomic study in *M. corti*, this data will constitute a high quality complement of the full genome sequence of the parasite, currently in its draft version.

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6. DISCUSSÃO E PERSPECTIVAS

Neste trabalho duas abordagens experimentais foram realizadas com o intuito de identificar moléculas reguladoras e genes associados aos mecanismos moleculares que acontecem em dois estágios do desenvolvimento do platelminto cestódeo modelo *Mesocestoides corti*.

A primeira abordagem experimental consistiu em uma análise compreensiva do repertório de miRNAs, uma classe de pequenos RNA não codificadores, no estágio larval (tetratirídeo) e verme estrobilado (segmentado), por meio de sequenciamento de nova geração. Um total de 42 miRNAs foram identificados, pertencentes a 33 famílias de miRNAs, o que confirma a perda de famílias de miRNAs conservadas dentro do filo Platyhelminthes. Foram identificados miRNAs específicos de cada estágio, assim como expressão diferencial de miRNAs, o que sugere que esses miRNAs estariam envolvidos na regulação de caraterísticas específicas de cada estágio no desenvolvimento. Por último, foi identificada a uridilação como mecanismo de modificação pós-transcricional nos miRNAs de *M. corti*.

A segunda abordagem experimental consistiu no estudo do transcritoma de tetratirídeos e vermes segmentados de *M. corti* com o intuito de identificar genes diferencialmente expressos entre ambos estágios do desenvolvimento. Foram identificados 342 genes sobre-expressos no tetratirídeo e 559 genes no verme estrobilado, adicionalmente 66 e 136 genes possuíam expressão estágio-específico, respectivamente. Dentro dos genes sobre-expressos em ambos estágios foram identificados genes codificadores de proteínas do citoesqueleto, proteases, proteínas com função antioxidante e do metabolismo de carboidratos e lipídeos, sugerindo que estes genes têm função imprescindível na manutenção de processos fisiológicos que acontecem em ambos estágios. Por outro lado, foram identificados genes sobre-expressos exclusivamente em um estágio ou outro, associados a processos estágio específico, como é a reprodução assexuada e migração do tetratirídeo nos tecidos do hospedeiro, e o desenvolvimento das gônadas e reprodução sexuada no verme estrobilado.

O primeiro capítulo deste trabalho descreve a caracterização do repertório de miRNAs de *M. corti*, mediante o sequenciamento de pequenos RNAs e posterior busca e anotação de miRNAs utilizando ferramentas de bioinformática.

Após, a expressão de vários dos miRNAs descritos foi validada por RT-qPCR em ambos os estágios de desenvolvimento estudados. A expressão diferencial de alguns destes miRNAs não puderam ser validados por RT-qPCR, como miR-36b, sobre-expresso no tetratirídeo, e miR-2a e miR-2b, sobre-expresso e estagío-específico do verme estrobilado, respectivamente. Uma caraterística dos miRNAs que pode influir na detecção destas moléculas, é a conformação de famílias com sequências similares (MARCO et al., 2012). Isto dificulta o desenho dos experimentos de PCR com uma eficiência suficiente para discernir entre integrantes de uma mesma família, como no caso da família miR-2 em *M. corti,* onde particularmente miR-2a e miR-2b possuem uma alta porcentagem de identidade, que difere em poucos nucleotídeos. Por outro lado, o processamento pós-transcricional dos miRNAs pode influenciar no alinhamento dos iniciadores de PCR (DELLETT; SIMPSON, 2016). Dado que miR-36b encontra-se uridilado no tetratirídeo, é possível que o isomiR-U deste miRNA pudesse influenciar no alinhamento dos iniciadores de RT-qPCR, por tanto dificultando a validação experimental nas amostras analisadas. Finalmente, o número de miRNAs a ser validado poderia ter sido maior, ficando como futura perspectiva deste trabalho.

No segundo capítulo, descreve-se a análise da expressão diferencial de genes entre o estágio larval e o verme estrobilado. Para isto foi realizado o sequenciamento de RNA total de cada estágio por triplicata biológica e a montagem dos transcritos utilizando a ferramenta Cufflinks (TRAPNELL et al., 2012). Como foi mencionado anteriormente, somente 50% das leituras obtidas foram mapeadas no genoma. As leituras que falharam em mapear podem constituir sequencias de baixa qualidade, contendo adaptadores ou erros no sequenciamento, e ainda podem constituir contaminantes introduzidos durante a preparação das livrarias ou no sequenciamento (TRAPNELL et al., 2012). A preparação correta das amostras constitui uma das etapas-chave para o sequenciamento de nova geração, onde a remoção de rRNA é importante para obter uma boa cobertura (BHAGWAT et al., 2014). Apesar de que as amostras foram tratadas com o kit Ribominus (Thermo) para a remoção do rRNA, e posteriormente analisadas por Bioanalyzer para constatar a eficiência do tratamento (ver Apêndice 9), foi identificada uma alta porcentagem de leituras correspondentes a rRNA nas amostras sequenciadas. Após a depleção *in silico* das leituras correspondentes a rRNA, foi observado um incremento da

porcentagem de leituras mapeadas a CDS (dados não mostrados). Uma alternativa a depleção de rRNA com o kit Ribominus, que mostrou não ser tão eficaz em *M. corti*, é a utilização de protocolos de enriquecimento de mRNA baseados em sondas oligo-dT (MORTAZAVI et al., 2008), eficientes na redução da quantidade de outras espécies de RNAs, como rRNA, tRNA e RNAs não codificadores.

Com os dados de expressão diferencial tanto de miRNAs como genes é possível especular sobre possíveis papeis regulatórios e processos moleculares associados a cada estágio do desenvolvimento. Dentre os miRNAs sobre-expressos em tetratirídeo encontra-se o miR-36b (Figure 4, capítulo 1). Em um estudo de predição de alvos de miRNAs na infecção experimental de *S. japonicum* em camundongos e ratos (HAN et al., 2015), miR-36 apresentou como mRNA-alvo, entre outros, o mRNA das proteínas calmodulina e calpaína. Ambas proteínas estão envolvidas em vias de sinalização por cálcio, em particular na via de sinalização Wnt/Ca^{2+} que regula a transcrição, via ativação do NFκB, de genes que controlam o destino e a migração celular (revisado em SONG et al., 2015). Tanto a calmodulina como a calpaína foram identificadas como sobre-expressas no tetratirídeo de *M.corti* na análise de expressão diferencial (ver Supplementary File 1, capítulo 2). A proteína Wnt4 de *S. japonicum* está envolvida na via clássica de sinalização Wnt/β-catenina (LI et al., 2010), porém tem sido demostrado que a sinalização via Wnt é complexa e integrada, com alta promiscuidade de ligandos e receptores, pudendo atuar em múltiplas vias (AMERONGEN, VAN; NUSSE, 2009). Por tanto, a sinalização por Wnt/Ca^{2+} poderia estar sendo regulada no tetratirídeo por meio da ação do miR-36b, com potencial papel regulador da transcrição na reprodução assexuada desta fase larvária.

miR-31 foi identificado como sobre-expresso no verme estrobilado. Este miRNA é um dos poucos miRNAs de platelmintos parasitos onde a interação com seu mRNA-alvo, o mRNA da proteína Frizz 7 (receptor da via de sinalização Wnt) e de uma o-glicosil-transferase, foi validada funcionalmente (*Schistosoma japonicum*; ZHU et al., 2016). A proteína Frizz 7 é um receptor pertencente à família de receptores acoplados a proteínas G (WINKLBAUER et al., 2001), que participa em múltiplas vias de sinalização, particularmente na modulação da atividade das proteínas Wnt. Estas proteínas estão envolvidas em vias de

sinalização nas etapas iniciais do desenvolvimento de platelmintos parasitos (Ll et al., 2010; RIDDIFORD; OLSON, 2011; KOZIOL et al., 2016). As glicosil-transferases são enzimas do metabolismo da glicose envolvidas na transferência de resíduos de açúcares de uma molécula doadora a uma aceptora (LAIRSON et al., 2008). O verme estrobilado tem uma alta taxa metabólica, associada à maturação sexual e a produção de ovos. Vários genes associados a Wnt e glicosil-transferases foram identificados como sobre-expressos no verme estrobilado, portanto, miR-31 poderia estar envolvido na regulação de processos relacionados a correta formação do estróbilo (a través da regulação da via Wnt) e as demandas energéticas associadas a reprodução sexuada (a través da regulação do transporte e metabolismo de carboidratos por glicosil-transferases) neste estágio do desenvolvimento.

Outro miRNA sobre-expresso no estágio de verme estrobilado é o miR-125. Vários alvos de miR-125 tem sido identificados, incluindo fatores de transcrição, genes das vias de apoptose entre outros. Em particular, miR-125 regula a somitogênese através da via de sinalização Notch (MOLONEY et al., 2000; AMBROS, 2011). Esta via de sinalização está presente em cestódeos, e ainda, foi identificado um gene codificante para a proteína delta 4 (pertencente à família lin-12/Notch) sobre-expresso no verme estrobilado. Em conjunto, isto poderia sugerir que miR-125 por meio da via Notch estaria regulando a transdução de sinais na maturação sexual do verme estrobilado.

Por tudo o que foi apresentado anteriormente, várias perspectivas de continuação do trabalho com miRNAs em *M. corti* são plausíveis: i) a busca e predição *in silico* de genes alvo dos miRNAs identificados como diferencialmente expressos em ambos estágios; ii) avaliação dos níveis transcricionais dos miRNAs e genes alvos, com o fim de identificar alguma correlação entre níveis de expressão entre o par miRNA-mRNA alvo; e por último, iii) realização de estudos funcionais da interação gene alvo-miRNA (ensaios funcionais por meio de ensaio de luciferase *in vitro* e experimentos de supressão de miRNAs *in vivo*). Ainda não existem alvos de miRNAs funcionalmente validados em cestódeos e *M. corti* constitui um bom modelo para a busca e validação destes mRNA-alvo.

As perspectivas de trabalho em relação à análise de genes diferencialmente expressos incluem: i) busca de ortólogos dos genes identificados em outras espécies de cestódeos, trematódeos e platelmintos de vida livre; ii) validação dos

dados de expressão diferencial *in silico* por RT-qPCR.

Em suma, neste trabalho foram identificadas moléculas regulatórias (miRNAs) e genes associados a dois estágios do desenvolvimento de *M. corti*. A informação gerada poderá ser base de estudos para determinar a função desses genes identificados e o papel regulador dos miRNAs nos processos moleculares que acontecem tanto no tetratirídeo, como é a reprodução assexuada por cissiparidade, como no verme estrobilado, como é o desenvolvimento do estróbilo e a reprodução sexuada.

Sendo *M. corti* um bom modelo de estudo da classe Cestoda pela disponibilidade de material biológico e facilidade de cultivo *in vitro*, alvos moleculares e mecanismos identificados neste parasito podem ser extrapolados para outros cestódeos e contribuir para o desenvolvimento de novos métodos de diagnóstico e tratamento de cestodíases. Vários alvos de drogas tem sido identificados em espécies do gênero *Echinococcus* por busca bioinformática no genoma e transcritoma (TSAI et al., 2013). Os critérios principais para a definição destes potenciais alvos de drogas anti-helmínticas incluem a expressão desse alvo no estágio do ciclo de vida desejado (por exemplo metacestódeo na hidatidose cística) e a disponibilidade de compostos ativos contra a classe molecular desse alvo (*druggability*). Dentre os genes identificados em *M. corti*, que foram identificados como potenciais alvos e que cumprem com os critérios anteriores, encontram-se genes que codificam para proteínas de sistemas de detoxificação (como a GST) expressos nos dois estágios analisados; receptores acoplados a proteínas G, como o Frizz7 (sobre-expresso no verme estrobilado) e canais de íons (sobre-expressos na fase larval). Outros alvos identificados em *M. corti* relevantes para o desenvolvimento de drogas contra cestódeos são as cinases (KOZIOL; BREHM, 2015), utilizadas atualmente para em estudos de *screening* de inibidores para o tratamento de esquistossomose (STROEHLEIN et al., 2015; LONG et al., 2016).

Adicionalmente, os miRNAs identificados em este estudo podem constituir marcadores para o diagnóstico da infecção parasitaria (BRITTON et al., 2015). Em concordância com isto, vários trabalhos tem identificado miRNAs de parasitos presentes no soro de hospedeiros infectados, demostrando o potencial dos miRNAs como biomarcadores. O sequenciamento de pequenos RNAs do soro de camundongos infectados com *Schistosoma mansoni* permitiu a

identificação de miRNAs do hospedeiro e do parasito (HOY et al., 2014), incluindo três miRNAs derivados do parasito que foram também identificados em *M corti* (bantam, miR-277 and miR-3479-3p). Além disso, alguns dos miRNAs secretados são empacotados em exossomos (vesículas extracelulares), como foi demostrado no trematódeo *F. hepatica* (CWIKLINSKI et al., 2015). No nosso grupo de trabalho foi reportada, por primeira vez em cestódeos, a presença de vesículas extracelulares no líquido que preenche o metacestódeo de *E. granulosus* (SANTOS, DOS et al., 2016), é possível pensar que os microRNAs de *M .corti* sejam secretados ao exterior via estas vesículas e por tanto possuam papel como biomarcadores da infecção. Por tanto, um estudo mais aprofundado dos origens e funções dos miRNAs expressados por *M. corti* poderá contribuir tanto no potencial diagnostico de outras cestoidíases, como no entendimento da interação parasito-hospedeiro.

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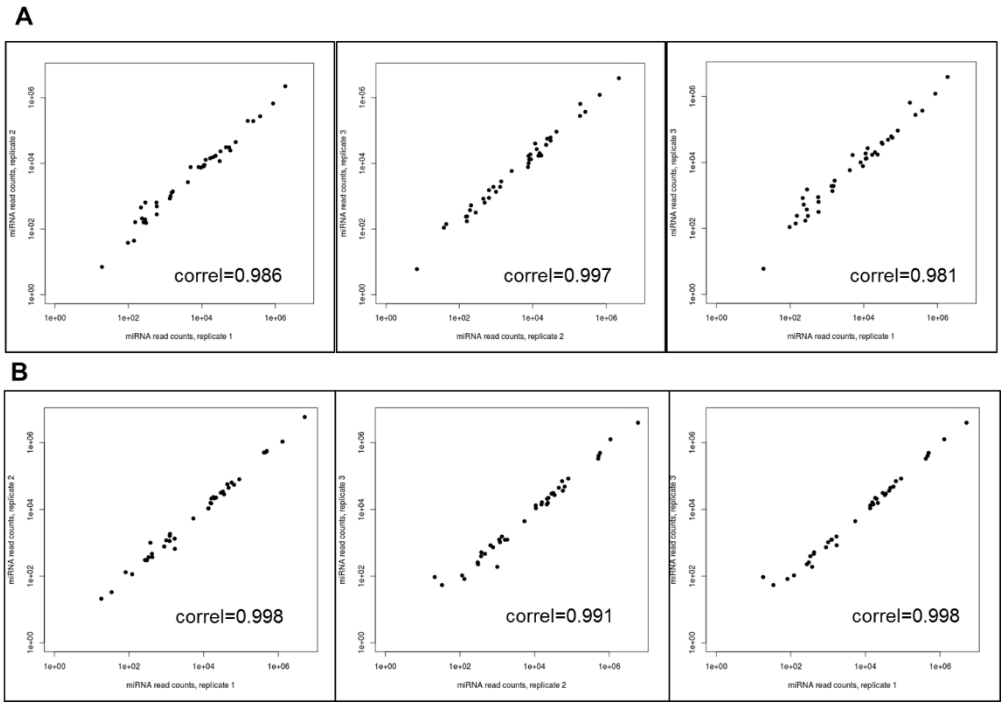
LISTA DE APÊNDICES

Apêndice 1 – disponível no arquivo Apêndice_1.pdf

Supplementary Figure 1 - Precursor structures of *Mesocestoides corti* microRNAs predicted with the reference genome. Red, mature microRNA; green, star microRNA; blue, products of both arms are considered mature; black, no mature product was detected.

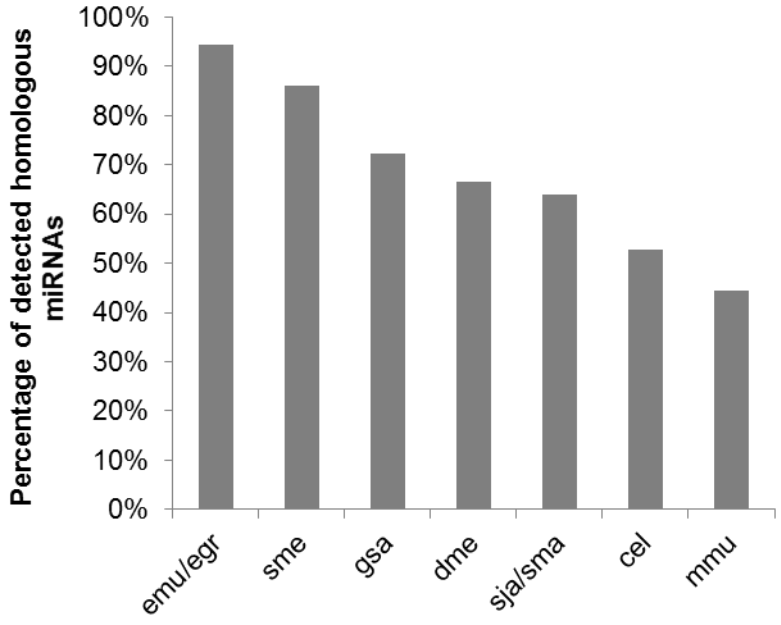
Apêndice 2 -



Supplementary Figure 2 – Correlation analysis between independent biological replicates of larvae and segmented worm of *Mesocestoides corti* – Each point represents one microRNA. Pearson correlation coefficients are shown in each plot. a) Tetrathyridia (TT), b) Strobilated worm (ST).

Apêndice 3 –

Species	N of detected homologous miRNAs	% of detected homologous miRNAs
E. multilocularis and *E. granulosus* (emu/egr)	34	94%
S. mediterranea (sme)	31	86%
G. salaris (gsa)	26	72%
D. melanogaster (dme)	24	66%
S. japonicum and *S. mansoni* (sja/sma)	23	64%
C. elegans (cel)	19	53%
M. musculus (mmu)	16	44%



Supplementary Figure 3 – Conservation of *Mesocestoides corti* miRNAs

Apêndice 4 – disponível no arquivo Apêndice_4.xlsx

Supplementary Table 1 – Primers used in Real Time PCR experiments.

Supplementary Table 2 - Endogenous gene evaluation for Real Time PCR experiments.

Supplementary Table 3 – Arm usage of *Mesocestoides corti* miRNAs.

Supplementary Table 4 – Precursor, star sequences and genomic localization of identified *Mesocestoides corti* miRNAs.

Supplementary Table 5 – Real time PCR validation of selected microRNAs expressed by tetrathyridia (TT) and strobilated worm (ST) stages of *Mesocestoides corti.*

Supplementary Table 6 - Real time PCR validation of selected stage biased microRNAs expressed by tetrathyridia (TT) and strobilated worm (ST) stages of *Mesocestoides corti*.

Supplementary Table 7 – IsomiR-U counts. Results are shown as average percentages (±S.D.) of biological replicates.

Apêndice 5 – disponível no arquivo Apêndice_5.xlsx

Supplementary File 1:

Supplementary Table 1A - upregulated genes in *Mesocestoides corti* tetrathyridia.

Supplementary Table 1B - upregulated genes in *Mesocestoides corti* strobilated worm.

Supplementary Table 1C - stage especific genes identified in *Mesocestoides corti* tetrathyridia.

Supplementary Table 1D - stage especific genes identified in *Mesocestoides corti* strobilated worm.

Apêndice 6 – disponível no arquivo Apêndice_6.xlsx

Supplementary File 2:

Supplementary Table 2A - GO term enrichment analysis for *Mesocestoides corti* tetrathyridia samples

Supplementary Table 2B - GO term enrichment analysis for *Mesocestoides corti* strobilated worm samples

Apêndice 7 – disponível no arquivo Apêndice_7.xlsx

Supplementary File 3:

Supplementary Table 3A - Biological Process GO terms for the genes upregulated and stage-specific in TT samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with grey colour.

Supplementary Table 3B - Cell Component GO terms for the genes upregulated and stage-specific in TT samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with pink colour.

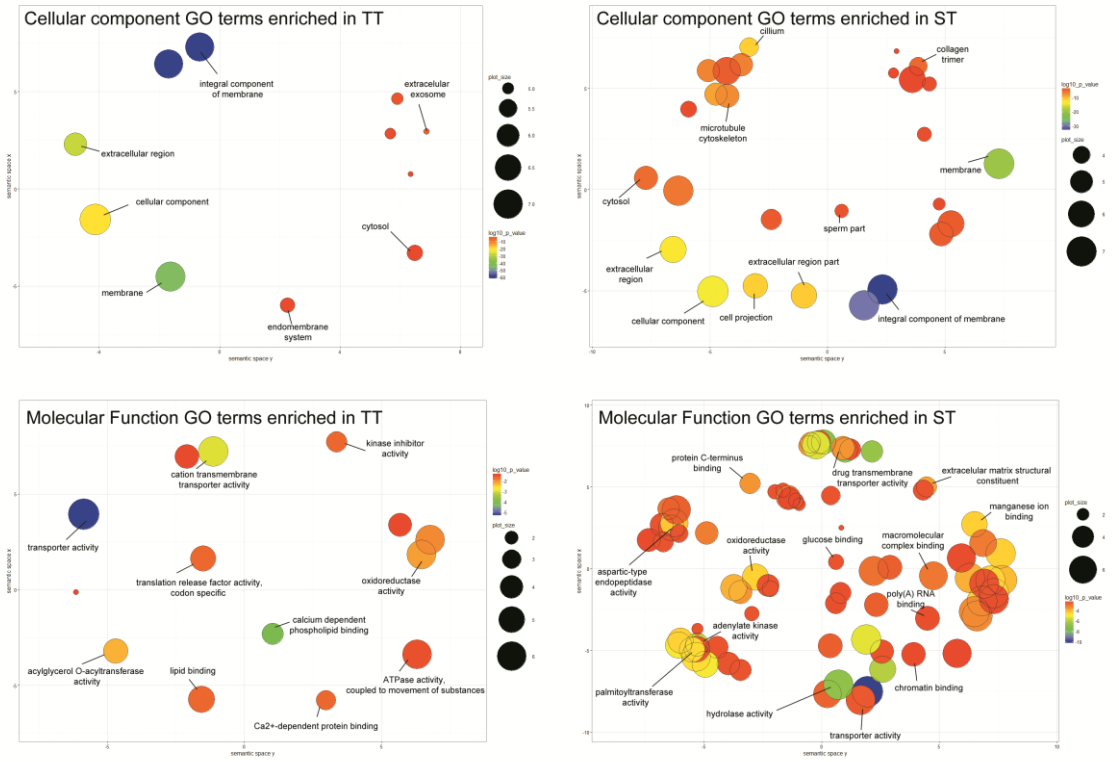
Supplementary Table 3C - Molecular Function GO terms for the genes upregulated and stage-specific in TT samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with light blue colour.

Supplementary Table 3D - Biological Process GO terms for the genes upregulated and stage-specific in ST samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with grey colour.

Supplementary Table 3E - Cell Component GO terms for the genes upregulated and stage-specific in ST samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with pink colour.

Supplementary Table 3F - Molecular Function GO terms for the genes upregulated and stage-specific in ST samples, summarised for semantic similarity using REVIGO. No colour indicates annotations summarised under those with light blue colour.

Apêndice 8 –



Supplementary File 4 – Summary of enriched GO terms for Cellular component and Molecular function – Scatter plot of the GO terms summarised by employing multidimensional scaling to the matrix of semantic similarities. Colour is proportional to the P-value obtained in the GO term enrichment analysis. Size is proportional to the term frequency in the GO annotation database, also indicating general terms. Names present the most significant terms in each group of semantically similar terms

Apêndice 9 – Análise da integridade do RNA extraído para a construção das livrarias de RNA

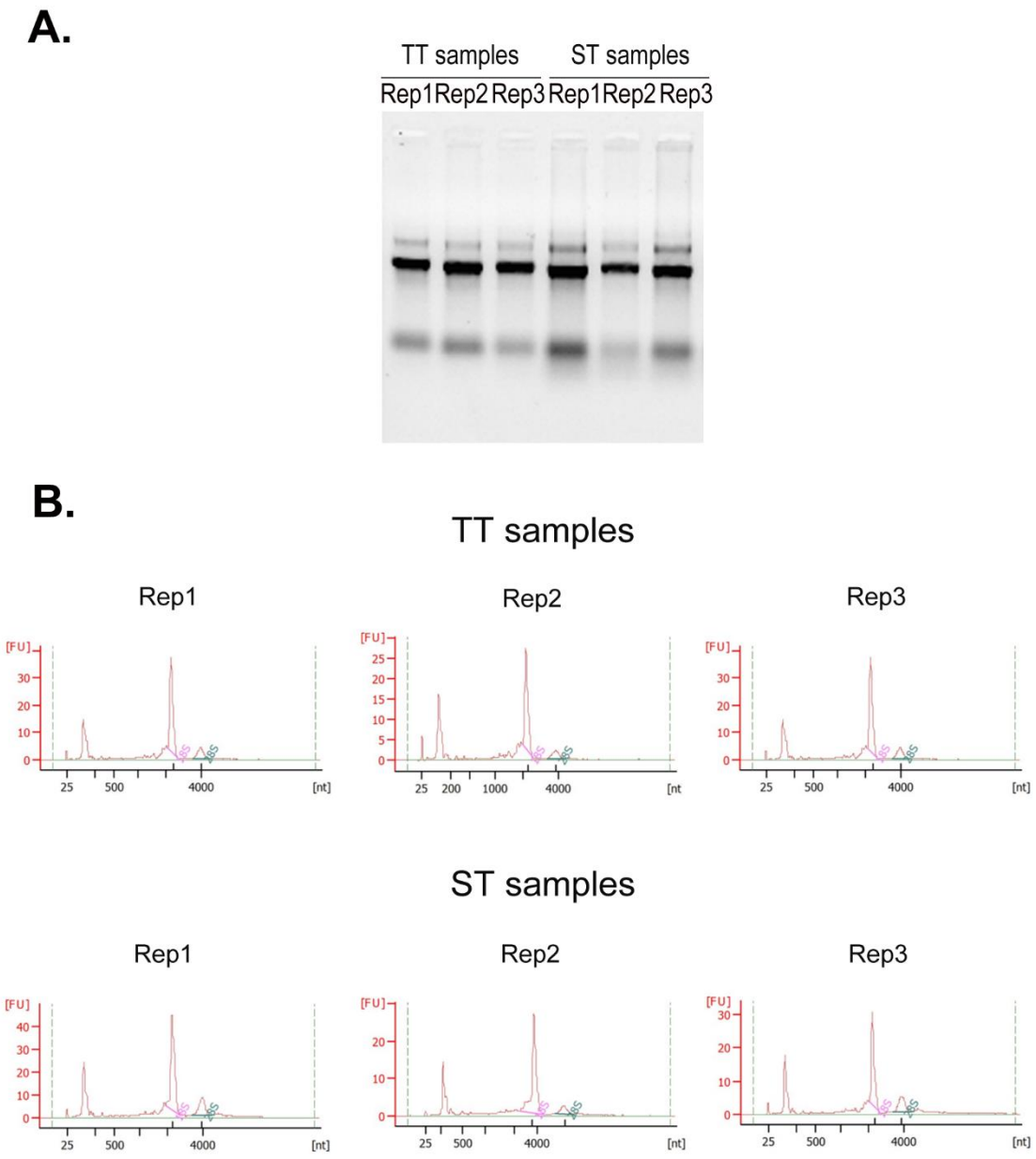


Figura 5 – Análise da integridade do RNA extraído de tetratirídeos e vermes estrobilados de *Mesocestoides corti*. A. Padrão electroforético do RNA extraído com o reagente Trizol em géis de agarose 0.8%. B. Electroferograma obtido no Agilent Bioanalyzer 2100. O RNA não apresenta sinais de degradação em nenhuma das técnicas utilizadas, e não há sinais de contaminação com DNA genômico, indicando que o RNA extraído possui uma boa qualidade.

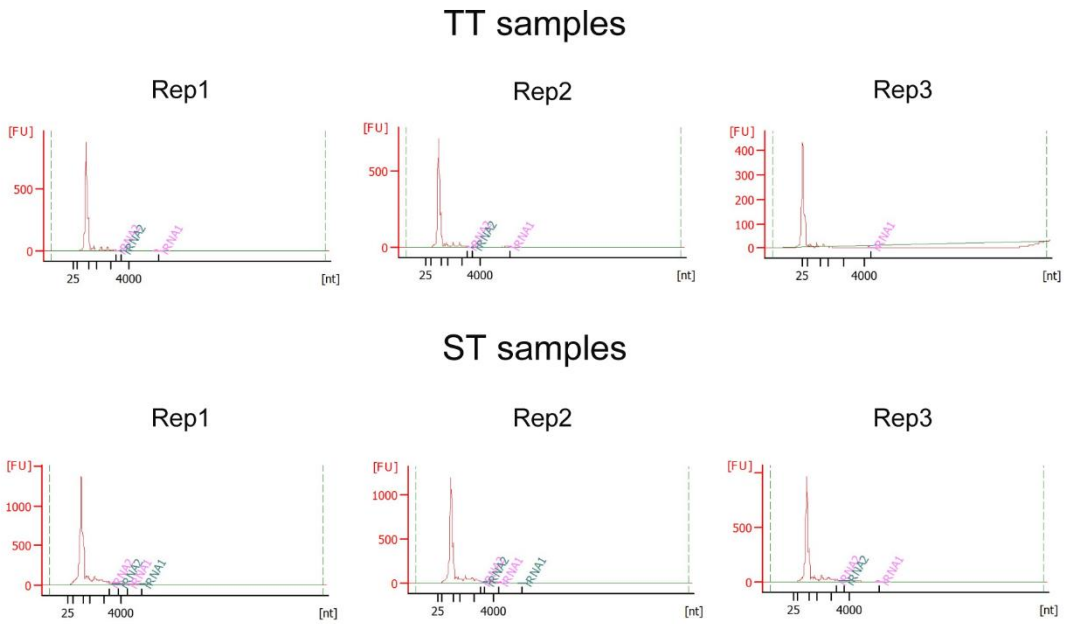


Figura 6 – Análise da integridade do RNA extraído de tetratirídeos e vermes estrobilados de *Mesocestoides corti* após a depleção de rRNA. Electroferograma obtido no Agilent Bioanalyzer 2100. O RNA não apresenta sinais de contaminação com rRNA em nenhuma das amostras, indicando que a remoção do rRNA foi bem sucedida.

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2. FORMAÇÃO ACADÊMICA:

2012-

Doutorado em andamento em Biologia Celular e Molecular (Conceito CAPES 6). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Título: Estudos transcritomicos do proceso de estrobilização do parasito cestódeo *Mesocestoides corti* (Cyclophyllidea, Mesocestoididae),

Orientador: Henrique Bunselmeyer Ferreira.

Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil

2009 – 2012

Mestrado em Biologia Celular e Molecular. Universidad de la Republica, UDELAR, Uruguai. Título: *Contribución a la dilucidación de los mecanismos proteolíticos que operan en la digestión intestinal de* Fasciola hepatica*.*

Orientador: Carlos Carmona García / Coorientador: José Francisco Tort.

Bolsista do(a): Agencia Nacional de Investigación e Inovación, ANII, Uruguai

2002 – 2008

Graduação em Licenciatura en Ciencias Biológicas. Facultad de Ciencias, UDELAR, Uruguai. Título: *Rol de las Catepsinas L1 y L2 de* Fasciola hepatica *en la degradación de hemoglobina*.

Orientador: Carlos Carmona García.

3. FORMAÇÃO COMPLEMENTAR

2013 -

Wellcome Trust Advanced Courses Working with Pathogen Genomes. (Carga horária: 44h). Sanger Center - Wellcome Trust Advanced Courses, SANGER, Uruguai.

2013 -

Introdução à técnica de interferência por RNA (RNA. (Carga horária: 80h). Universidade de São Paulo, USP, Brasil.

2012 -

Generation of Libraries for next generation sequencing. (Carga horária: 64h). Instituto Pasteur de Montevideo, PASTEUR, Uruguai.

2010

Mass spectrometry in protein analysis (Carga horária: 40h). Instituto Pasteur de Montevideo, PASTEUR, Uruguai

4. ESTÁGIOS

2015 -

Bolsa doutorado sanduiche (duração 3 meses). Analise do transcritoma de *Mesocestoides corti.* Projeto CAPES/CAPG. Universidad de Buenos Aires, Argentina.

Responsável: Dra, Laura Kamenetzky

2014 –

Bolsa doutorado sanduiche (duração 1 mês). Caraterização de microRNAs de *Mesocestoides corti*. Projeto CAPES/Mincyt. Universidad de Buenos Aires, Argentina.

Responsável: Dra, Mara Rosenzvit

2011 –

Bolsa PEDECIBA (duração 2 meses). Missão de estudo do mestrado. University of California, San Francisco, Estados Unidos.

Responsável: Dr. Connor Caffrey

2009 –

Bolsa Jovenes Investigadores (duração 4 meses). *Análisis de proteínas de tegumentos de Fasciola hepática involucradas en la respuesta del hospedador ovino*. Universitat de Valencia, España.

Responsável: Dr. Antonio Marcilla

5. EXPERIÊNCIA PROFISSIONAL E DIDÁTICA ANTERIOR

2011-2012

Ajudante de Pesquisa. Projeto CSIC "*Genómica funcional del desarrollo de platelmintos parásitos*", Departamento de Genética, Facultad de Medicina, UdelaR

2009- 2011

Ajudante de Pesquisa. Projeto CSIC "*Digestión de la hemoglobina por* Fasciola hepatica*: dilucidación de la cascada proteolítica e identificación de nuevos blancos moleculares con potencial aplicación al control de la parasitosis*", Unidad de Biología Parasitaria, Facultad de Ciencias, UdelaR.

2007-2008

Ajudante de Pesquisa. Projeto PDT "*Proteínas S100 y anexinas extracelulares en la dicotomía resolución versus cronicidad inflamatoria en la hidatidosis*", Cátedra de Inmunología, Facultad de química, UdelaR.

2015

Práticas de Laboratório II. Centro de Biotecnologia da UFRGS, Instituo de Biociencias, Universidade Federal do Rio Grande do Sul. Carga horária: 30 horas. Prof. responsável: Karina M. Monteiro.

2012

Proteomas de Parasitos. Fundamentos y Aplicaciones. Instituto de Higiene, Facultad de Ciencias, UdelaR. Carga horária: 30 horas. Prof. responsáveis: Dra. Patricia Berasain, Dr. Charley Staats.

2007- 2010

Seminarios de Introducción a la Biología. Facultad de Ciencias, UdelaR. Carga horária: 30 horas. Docente responsable Dra. Patricia Berasain.

2007- 2010

Biologia Parasitaria. PEDECIBA Biologia, UdelaR. Carga horária: 30 horas. Coordenador Dr. Carlos Carmona. Prof. responsável Dra. Patricia Berasain.

6. ARTIGOS COMPLETOS PUBLICADOS

BASIKA, T., MACCHIAROLI, N., CUCHER, M., ESPÍNOLA, S., KAMENETZKY, L., ZAHA, A., ROSENZVIT, M., FERREIRA, H.B., 2016. Identification and profiling of microRNAs in two developmental stages of the model cestode parasite *Mesocestoides corti*. Mol. Biochem. Parasitol. doi:10.1016/j.molbiopara.2016.08.004

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glutathione reductase failed to confer protection against fasciolosis in cattle. Vet. Parasitol. 224, 13–19. doi:10.1016/j.vetpar.2016.05.007

DELL'OCA, N., **BASIKA, T**., CORVO, I., CASTILLO, E., BRINDLEY, P.J., RINALDI, G., TORT, J.F., 2014. RNA interference in *Fasciola hepatica* newly excysted juveniles: Long dsRNA induces more persistent silencing than siRNA. Mol. Biochem. Parasitol. 197, 28–35. doi:10.1016/j.molbiopara.2014.10.001

BASIKA, T., MUÑOZ, N., CASARAVILLA, C., IRIGOÍN, F., BATTHYÁNY, C., BONILLA, M., SALINAS, G., PACHECO, J.P., ROTH, J., DURÁN, R., DÍAZ, A., 2012. Phagocyte-specific S100 proteins in the local response to the *Echinococcus granulosus* larva. Parasitology 139, 271–283. doi:10.1017/S003118201100179X

ROSS, F., HERNÁNDEZ, P., PORCAL, W., LÓPEZ, G.V., CERECETTO, H., GONZÁLEZ, M., **BASIKA, T.**, CARMONA, C., FLÓ, M., MAGGIOLI, G., BONILLA, M., GLADYSHEV, V.N., BOIANI, M., SALINAS, G., 2012. Identification of thioredoxin glutathione reductase inhibitors that kill cestode and trematode parasites. PLoS One 7. doi:10.1371/journal.pone.0035033

MAGGIOLI, G., ACOSTA, D., SILVEIRA, F., ROSSI, S., GIACAMAN, S., **BASIKA, T**., GAYO, V., ROSADILLA, D., ROCHE, L., TORT, J., CARMONA, C., 2011. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine 29, 9057–63. doi:10.1016/j.vaccine.2011.09.020