Universidade do Estado do Rio de Janeiro

Centro Biomédico

Faculdade de Ciências Médicas

Joyce Gonçalves Rozário de Souza

O modelo *Echinostoma paraensei* (Trematoda:Digenea): avaliação do grau de compatibilidade entre hamster e rato Wistar, características do desencistamento *in vitro* e das alterações morfológicas provocadas pelo artesunato

Rio de Janeiro

2015

Joyce Gonçalves Rozário de Souza

O modelo *Echinostoma paraensei* (Trematoda:Digenea): avaliação do grau de compatibilidade entre hamster e rato Wistar, características do desencistamento in vitro e das alterações morfológicas provocadas pelo artesunato

Tese apresentada, como requisito parcial para obtenção do título de Doutor , ao Programa de Pós-graduação em Microbiologia, da Universidade do Estado do Rio de Janeiro. Área de concentração: Microbiologia Médica Humana.

Orientador: Prof. Dr. José Roberto Machado e Silva
Coorientador: Dr. Arnaldo Maldonado Junior

Rio de Janeiro

2015

Autorizo apenas para fins acadêmicos e científicos, a reprodução total ou parcial desta tese, desde que citada a fonte.

_____   _____

Assinatura   Data

Joyce Gonçalves Rozário de Souza

O modelo *Echinostoma paraensei* (Trematoda:Digenea): avaliação do grau de compatibilidade entre hamster e rato Wistar, características do desencistamento in vitro e das alterações morfológicas provocadas pelo artesunato

<div align="right">

Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-graduação em Microbiologia, da Universidade do Estado do Rio de Janeiro. Área de concentração: Microbiologia Médica Humana.

</div>

Aprovada em 27 de fevereiro de 2015.

Coorientador: Dr. Arnaldo Maldonado Junior
                        Fundação Oswaldo Cruz

Banca Examinadora:

_____
Prof. Dr. José Roberto Machado e Silva (Orientador)
Faculdade de Ciências Médicas – UERJ

_____
Prof.ª Dra. Renata Heisler Neves
Faculdade de Ciências Médicas – UERJ

_____
Prof. Dr. Válber da Silva Frutuoso
Fundação Oswaldo Cruz

_____
Prof.ª Dra. Simone Chinicz Cohen
Fundação Oswaldo Cruz

_____
Prof.ª Dra. Claudia Maria Uchôa Souto Maior
Universidade Federal Fluminense

Rio de Janeiro
2015

Dedico esta conquista à minha amada filha Júlia Gonçalves Vieira que me veio como um presente no início do segundo ano de doutorado. Que você filha um dia entenda a substituição que eu fiz do meu tempo com você por este doutorado. Ele foi também em sua função.

AGRADECIMENTOS

Aos meus orientadores Prof. Dr. José Roberto Machado e Silva pelo exemplo profissional, pelo crédito depositado em minha pessoa e iniciação à carreira científica e ao Dr. Arnaldo Maldonado Junior pela dedicação em ensinar, estímulos e críticas. Aos dois, os meus profundos agradecimentos por tudo que fizeram por mim durante todo esse tempo, contribuindo para o meu crescimento acadêmico e humano. Obrigada pela preocupação, dedicação, paciência e apoio que levaram a concretização deste trabalho. Espero um dia poder retribuir.

Aos professores da Pós-graduação em Microbiologia Médica Humana pela contribuição ao meu conhecimento em Microbiologia, Parasitologia e Imunologia e incentivo a pesquisa.

Ao tecnologista Dr. Juberlan Silva Garcia da FIOCRUZ pelos estímulos, positividade, amizade, preocupação, disponibilidade em ajudar, conversar, discutir ideias e pela divisão das tarefas práticas experimentais. À você amigo Juberlan, o meu muito obrigada por sempre estar por perto, sem a sua ajuda tudo seria mais árduo.

À Dra. Rosângela Rodrigues e Silva e à Dra. Fernanda Almeida Barbosa, ambas da FIOCRUZ, pelas mãos estendidas e pela preocupação com o andamento e finalização desta tese. Dra Rosângela, obrigada por permitir que eu fizesse parte da família "Parasitos de Vertebrados", pela confiança, pela porta aberta e "liberdade" em usar o laboratório. Dra. Fernanda, o meu obrigada por sempre estar interessada em dar tudo certo, em resolver problemas para que eu pudesse finalizar a parte prática, apoio e pelo pensamento sempre positivo. Muito obrigada!

Ao Prof. Dr. Eduardo Lopes Torres da UERJ, pela dedicação em ensinar o que sabe, pelas inúmeras dicas, correções e por estar sempre se disponibilizando a ajudar. A você a minha admiração e gratidão.

À Dra Renata Heisler pelas dicas, ajuda, convivência e por aceitar e revisar esta tese.

Aos amigos do LBPMR pelo convívio de anos, pela descontração, preocupação e ajuda. Sem o bom humor dessas pessoas o meu caminhar seria mais difícil. Obrigada Michele Maria, Neto, Jeiel, Sandra, Juliana Ferraz, Juliana São Luis, Tainá, Bernardo, André Santana, Marta Júlia, Mayara, Camila, Fernanda e Tatiane.

Aos amigos da família UERJ, do Laboratório de Hemintologia Romero Lascasas Porto, onde aprendi a ser mais solidária e vi que a união dentro de um laboratório transforma

e deve existir. Obrigada Vanessa, Michele Costa, Luciana, Fred, Alba, Renata Heisler, Regina, Thaís, Suellen e Bruno.

Aos amigos do Laboratório de Helmintos Parasitos de Vertebrados: Fernanda Bittencourt, Jessica, Simone, Daniel, Leandro, Natalia e Tuan.

À Universidade do Estado do Rio de Janeiro (UERJ), onde fiz toda a minha formação acadêmica durante os últimos 12 anos, graduação, mestrado e doutorado.

À Fundação Oswaldo Cruz (FIOCRUZ), Instituto Oswaldo Cruz (IOC) por oferecer espaço, infra-estrutura, possibilidade de pesquisa bibliográfica, aperfeiçoamento acadêmico, desenvolvimento de técnicas de metodologia científica e pela grande contribuição no conhecimento de pesquisas avançadas na área médica.

Ao meu pai, veterinário, e à minha mãe, professora, de quem eu herdei o gosto pela ciência e o prazer da licenciatura, respectivamente. Àquelas pessoas que eu tenho certeza que são os meus fãs número 1, obrigada pela torcida, crédito e incentivo.

Ao meu companheiro Alexandre por me substituir em casa e com a Júlia, por compreender a substituição do nosso tempo pelo doutorado e por sempre me encorajar e me dizer: vai dar tudo certo!

À minha irmã Jane e ao meu irmão Marcus Paulo pela torcida.

Aos colegas e demais funcionários tanto da FIOCRUZ quanto da UERJ por colaborar com a realização deste trabalho e pelo apoio dado de uma forma geral.

A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.

Albert Eintein

RESUMO

SOUZA, Joyce Gonçalves Rozário de. O modelo *Echinostoma paraensei* (Trematoda:Digenea): avaliação do grau de compatibilidade entre hamster e rato Wistar, características do desencistamento *in vitro* e das alterações morfológicas provocadas pelo artesunato. 2015. 148f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2014.

     Echinostoma paraensei é reconhecido como modelo adequado, entre outros fatores, face à facilidade de manutenção do ciclo em laboratório. Pouco se conhece sobre a cinética de desenvolvimento em hospedeiros com diferentes graus de compatibilidade, dos fatores envolvidos no desencistamento e a suscetibilidade dessas formas ao tratamento com fitoterápico. Nessa tese esses aspectos foram analisados. Metacercárias de *E. paraensei* foram removidas de *Biomphalaria glabrata* e incubadas *in vitro* em meio de tripsina e sais alcalinos para o estudo do desencistamento. Esse dependeu da presença de sais de bile e tripsina, mas não das concentração estudadas, atingindo altas taxas ao final de 2 horas. Por meio de microscopia de luz foram verificados quatro estágios de desenvolvimento: encistado, ativado, rompido e desencistado, semelhante ao descrito para outras espécies do gênero. Por microscopia de varredura laser confocal foi demontrado que as formas recém desencistadas apresentaram morfologia similar aos helmintos adultos, com exceção das estruturas do sistema reprodutor, nas quais estão presentes como primórdios genitais. Hamsters e ratos albinos foram infectados por gavagem e necropsiados com 3, 7, 14 e 21 dias de infecção. Por microscopia de campo claro foi observada maior taxa de recuperação de vermes em hamster, principalmente, nas duas últimas semanas. Nesse hospedeiro houve maior taxa de fecundidade, representado por maior número de espécimes ovígeras. Ao contrário, os dados morfométricos das gonadas eram significativamente menores em ratos. Por microscopia confocal foi obervado ovário atrofiado, espécimes com ausência de glândula vitelínica, embora houvesse espermiogênese e espermatozóides na vesícula seminal. Os dados acrescentam novas informações sobre o hamster como hospedeiro compatível do *E. paraensei*. O estudo dos aspectos fenotípicos de helmintos incubados *in vitro* é um método indicado para verificar o desenvolvimento de atividades terapêuticas. Metacercárias foram mantidas em meio RPMI 1640 com 1, 10, 25, 50 e 100 µg/ml de artesunato. A partir da concentração de 25 µg/ml do fármaco houve aumento da taxa de mortalidade e diminuição progressiva dos movimentos. As concentrações 100 µg/mL e 50 µg/mL causaram efeito deletério após 24 e 48 h, respectivamente. Por microscopia eletrônica de varredura foi verificado que os helmintos incubados com 50 e 100 µg/mL de artesunato, apresentaram inchaço, vesículas, orifícios, erupções, dobras no tegumento e perda de espinhos do collar. Pela primeira vez, foi demonstrado que larvas desencistadas são suscetíveis ao tratamento. O conjunto de dados confirma que *E. paraensei* é um modelo apropriado para estudos *in vitro* ou *in vivo* da biologia de trematódeos. As facilidades de cultivo *in vitro*, apontam para a possibilidade do estabelecimento de métodos alternativos nos estudos sobre o desenvolvimento e testagem de fármacos de origem vegetal com potencial de atividade antihelmíntica. Do ponto de vista ético, o cultivo *in vitro* poderá contribuir para a redução na utilização de animais nas fases iniciais do teste dos fármacos.

Palavras-chave: *Echinostoma paraensei*. Desencistamento. *In vitro*. Hamster. Rato. Estudo morfológico. Morfometria. Artesunato. Microscopia de luz de campo claro. Microscopia eletrônica de varredura confocal. Microscopia eletrônica de varredura.

ABSTRACT

SOUZA, Joyce Gonçalves Rozário de. The *Echinostoma paraensei* (Trematoda : Digenea) model: evaluation of the degree of compatibility between hamster and Wistar rat, *in vitro* excystation characteristics and morphological changes caused by artesunate. 2015. 148f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2014.

Echinostoma paraensei is recognized as an appropriate model, among other factors, due to ease of maintenance in the laboratory cycle. Little is known about the development kinetics of hosts with different degrees of compatibility, the factors involved on excystation and the susceptibility of these stages to treatment with herbal medicine. In this thesis these aspects were analyzed. Metacercariae of *E. paraensei* were removed from *Biomphalaria glabrata* and incubated *in vitro* in Alkaline trypsin bile salts for excystation study. The excystation depended on the presence of trypsin and bile salts, but not of concentrations studied, reaching high levels after 2 hours. By light microscopy were verified four stages of development: encysted, activated, breached and excysted, similar to that described for other species of the genus. By confocal laser scanning microscopy was played that newly excysted metacercariae showed a similar morphology to adults helminths, with the exception of the reproductive system structures in which are present as genital anlage. Albino rats and hamsters were infected by gavage and necropsied at 3, 7, 14 and 21 days after infection. By light microscopy were verified highest worm recovery rate in hamster, especially in the last two weeks. In this host was higher fertility rate, represented by greater number of ovigerous specimens. Rather, morphometric data of the gonads were significantly lower in rats. By confocal microscopy was observed atrophied ovaries, specimens with no vitelline glands, although there was spermiogenesis and spermatozoa in the seminal vesicle. The data add new information about the hamster as compatible host of *E. paraensei*. The study of phenotypic aspects of helminth incubated *in vitro* is a suitable method to monitor the development of therapeutic activities. Metacercariae were maintained in RPMI 1640 with 1, 10, 25, 50 and 100 µg/mL artesunate. At concentrations 25 µg/ mL of the drug there was increased of mortality rate and progressive reduction of movements. The concentrations of 100 µg/mL and 50 µg/mL showed deleterious effects after 24 and 48 h, respectively. For scanning electron microscopy was found that the helminths incubated with 50 and 100 µg /mL of artesunate showed swelling, blebs, holes, eruptions, folds on the integument and loss of collar spines. For the first time it was shown that excysted larvae are susceptible to treatment. The data set confirms that *E. paraensei* is an appropriate model for *in vitro* studies or *in vivo* trematodes biology. *In vitro* culture of the facilities, point to the possibility of providing alternative approaches to the study of development and testing of plant-derived drugs with potential anthelmintic activity. From an ethical point of view, the *in vitro* culture can help to reduce the use of animals in the early stages of testing of drugs.

Keywords: *Echinostoma paraensei*. Excystation. *In vitro*. Hamster. Rat. Morphological study. Morphometry. Artesunate. Light microscopy.Laser scanning confocal microscopy. Scanning electron microscopy.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

Ac	Acetábulo
AFA	Ácido acético, formalina e álcool
AT	Anterior testis (testículo anterior)
B	Blebs (vesículas)
BC	Bolsa do cirro
BED	Bulbo of ejaculatory duct (bulbo do ducto ejaculatório)
C	Ceco
CC	Calcareous corpuscles (corpúsculos calcáreos)
CI	Cirro
CM	Circular muscles (músculo circular)
CEUA	Comitê de ética no uso animais
CL	Canal de Laurer
CP	Colar peristomático
CS	Cirrus'sac (bolsa do cirro)
CV	Células vitelínicas
DE	Ducto ejaculatório
DM	Diagonal muscles (músculos diagonais)
DV	Ducto vitelínico
dpi	Dia posterior à infecção/ Days post infection
E	Espermatozóides
EC	Excretory concrection (concreção excretora)
ED	Ejaculatory duct (ducto ejaculatório)
EP	Empty pocket ("pacote" vazio)
F	Faringe
GA	Genital anlage (primórdios genitais)
GM	Glândula de Mehlis
GP	Gonoporo
GV	Glândula vitelínica
LASER	Light Amplification by Stimulated Emission
LC	Laurer's canal
LM	Longitudinal muscles (músculos longitudinais)

IC	Immature cells (células imaturas)
ICW	Inner Cist Wall (parede interna do cisto)
MCC	Microscopia de campo claro
MLC	Microscopia Laser Confocal
MET	Microscopia Eletrônica deTransmissão
MEV	Microscopia Eletrônica de Varredura
MG	Mehlis'gland (glândulas de Mehlis)
MM	Meridional muscles (músculo meridional)
O	Ovo
OCW	Outer Cist Wall (parede externa do cisto)
OO	Oótipo
OOC	Oócitos
OV	Ovário
OVC	Ovicapt
OVD	Oviducto/ oviduct
OVV	Ducto ovovitelínico
OS	Oral sucker
P	Poro
PC	Peristomic collar (colar peristomático)
PE	Parenchimal extrusion (extrusão do parênquima)
PG	Primórdio genital
PT	Posterior testis (testículo posterior)
RM	Radial muscles (músculo radial)
RS	Receptáculo seminal
RV	Reservatório vitelínico
S	Swelling (inchaço)
SV	Seminal vesicle
SP	Sperm (espermatozóide)
SR	Seminal Receptacle (receptáculo seminal)
TA	Testículo anterior
TP	Testículo posterior
TS	Tegumental spine (espinho tegumentar)
VD	Vitelline duct (ducto vitelínico)

VF	Vitelline follicle (folículo vitelínico)
VO	Ventosa oral
VV	Ventosa ventral
VG	Vitelline glands (glândulas vitelínicas)
VR	Vitelline reservoir (reservatório vitelínico)
VS	Vesícula seminal
U	Útero

SUMÁRIO

INTRODUÇÃO

Classificação Taxonômica

Filo Platyhelminthes

    Classe Trematoda (Rudolphi, 1808)

        Subclasse Digenea (Carus, 1863)

            Família Echinostomatidae (Looss, 1899)

                Gênero Echinostoma (Rudolphi, 1809)

                    Echinostoma paraensei (Lie & Basch, 1967)

A classe Trematoda é dividida em duas subclasses: Aspidogastrea e Digenea. Esta última caracteriza-se por apresentar helmintos com alternância de gerações, cuja maioria utiliza um molusco como hospedeiro intermediário e um vertebrado como hospedeiro definitivo. Em geral, os helmintos são hermafroditas, de corpo alongado-oval para tubular ou arredondado e ainda filamentoso. Usualmente apresentam duas ventosas ou uma e ocasionalmente nenhuma. A boca é circundada pela ventosa oral, a pré-faringe pode estar ausente e a faringe sempre presente. O intestino é bifurcado e raramente ausente. A forma dos testículos é variável, bolsa do cirro é frequente, podendo estar toda ou apenas uma parte envolvida pela genitália feminina. O poro genital está normalmente presente e sua posição é variável (ventral-anterior e ocasionalmente na região posterior do corpo). Ovário normalmente único cuja posição e forma são variáveis. O útero e glândulas vitelínicas de tamanho e distribuição variável. O poro excretor é único, normalmente terminal ou subterminal conectado a vesícula sacular que pode apresentar a forma de I, V ou Y. Os helmintos da classe Digenea estão em sua grande maioria presentes no intestino e outras cavidades, no entanto podem ser também encontrados no sangue e outros tecidos (Kostadinova & Gibson, 2000).

Os representantes da família Echinostomatidae possuem ampla distribuição geográfica parasitando aves, répteis, peixes e mamíferos silvestres e domésticos, incluindo o homem (Kostadinova & Gibson, 2000). Tem como gênero tipo *Echinostoma*. Muitos possuem hábitos hematófagos, o que os tornam parasitos de relevante importância médica e econômica. Costumam infectar a maioria dos órgãos da cavidade abdominal, como os dos sistemas

digestivo, excretor e reprodutor. A manutenção do ciclo de algumas espécies dessa família tem servido como modelo experimental por ser de fácil manutenção e pouco onerosa (Fried &Toledo, 2009).

O gênero *Echinostoma* é o mais antigo e maior gênero em termos de número de espécies descritas. Constitui um grupo importante de helmintos por parasitar animais domésticos, silvestres e ocasionalmente, o homem (Fried & Grazyck, 2004). Apresenta como espécie tipo *Echinostoma revolutum* (Froelich, 1802) Dietz, 1909, descrito a partir de material obtido de moluscos naturalmente infectados, na Alemanha. De todas as características de identificação do gênero *Echinostoma*, as mais importantes são número, tamanho, forma e arranjo do colar de espinhos localizados na ventosa oral desses digenéticos. Mais de 100 espécies de *Echinostoma* foram descritas (Kanev, 1990), entretanto há controvérsia em relação às características dos espinhos que essas espécies descritas apresentam (Kanev et al., 2009). Recentemente, com base em dados morfológicos e moleculares, foi realizada uma detalhada revisão das espécies européias do gênero (Georgieva et al., 2014; Faltýnková et al., 2015). Esses estudos revelaram a presença de *E. revolutum* (sensu stricto), *E. miyagawai*, *E. paraulum*, *E. bolschewense* and *Echinostoma* n. sp.

Histórico da espécie

O trematódeo digenético *Echinostoma paraensei* Lie e Basch, (1967) foi descrito, a partir do isolamento de cercárias presentes em *Biomphalaria glabrata* coletada no Estado de Minas Geraisl. O molusco naturalmente infectado foi transportado para os Estados Unidos, onde o ciclo experimental foi passado em hamsters, ratos albinos e camundongos, possibilitando a obtenção e descrição, não somente, do helminto adulto, como das demais formas evolutivas deste parasito. No entanto, o hospedeiro definitivo natural permaneceu desconhecido.

No Brasil, Maldonado et al (2001a) identificaram o roedor *Nectomys squamipes* (Figura 1) como hospedeiro definitivo natural de *E. paraensei* e propuseram um ciclo biológico para este parasito. A principal característica taxonômica está presente no colar peristomático com 37 espinhos, cujo número e a disposição são utilizados para diferenciar as espécies. A redescrição da espécie por Maldonado et al (2001a) foi baseada em microscopia de luz de campo claro, microscopia eletrônica de varredura e técnicas de biologia molecular. Desde essa redescrição o ciclo biológico deste parasito tem sido mantido no Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, na Fundação Oswaldo Cruz, Rio de

Janeiro, e tem sido fonte de material biológico para estudos em diferentes subáreas tais como a taxonomia (Maldonado et al., 2001a, Maldonado et al., 2003), descrições morfológicas de diferentes formas evolutivas (Pinheiro et al., 2004, Pinheiro et al., 2005; Souza et al., 2011), interação parasito-hospedeiro (vertebrado e invertebrado) (Maldonado et al., 2005; Garcia et al., 2011), mudanças fisiológicas (Maldonado et al., 2001b, Pinheiro et al., 2009; Tunholi et al., 2011a,b,c; Tunholi-Alves et al., 2011)), associação com outros helmintos (Garcia et al., 2011) e teste de drogas (Ferraz et al., 2012; Gonçalves et al., 2013).

Figura 1 - O roedor *Nectomys squamipes*, hospedeiro definitivo de *Echinostoma paraensei*

Fonte: Sócrates F. C. Neto

Morfologia de *Echinostoma paraensei*

Echinostoma paraensei (Figura 2) é um típico trematódeo hermafrodita, apresenta o corpo alongado e fino com um colar peristomático ao redor da ventosa oral, cujo número e disposição dos espinhos são utilizados como caracteres taxonômicos. O colar pode apresentar 35, 36 ou 37 espinhos, sendo distribuídos dorso, lateral e ventro-lateral, e ainda interrompido na região ventral. A ventosa oral é muscular e subterminal seguida de uma pré-faringe curta e a faringe também muscular e bulbar. O esôfago é longo e se bifurca anteriormente ao acetábulo formando dois cecos que seguem lateralmente pelo corpo do trematódeo. A ventosa ventral é desprovida de espinhos, muscular e maior que a ventosa oral. O poro excretor é subterminal localizado na região posterior. Sistema reprodutor composto por útero contorcido, um ovário ovóide, dois testículos bilobados, bolsa do cirro com vesícula seminal, glândula prostática e cirro. Glândulas vitelínicas localizados nas laterais do corpo. As glândulas de Mehlis estão localizadas ao redor do oótipo (Maldonado et al., 2001a).

O sistema reprodutor feminino apresenta um ovário localizado anterior ao testículo. O oviducto parte do ovário e ainda próximo a ele junta-se a uma estrutura na forma de bulbo chamada receptáculo seminal, cuja função é estocar espermatozoides. As glândulas vitelínicas ou vitelárias são estruturas emparelhadas localizadas na lateral do corpo e comunica-se com o oviducto por meio dos ductos vitelínicos que partem de cada lado do corpo; algumas espécies possuem um alargamento nesta região chamado de reservatório vitelínico. O oviducto possui uma dilatação cercada por glândulas (glândulas de Mehlis) que se refere ao oótipo ou câmara de fertilização. Um canal, presente em algumas espécies, deixa o oviducto e se abre para superfície do corpo por um poro. A este canal dá-se o nome de canal de Laurer. Acredita-se que esse canal seja homólogo a vagina de cestóides, terminando cegamente em algumas espécies. A função do canal do canal de Laurer ainda não foi esclarecida. O oótipo se abre dentro de um longo e frequentemente contorcido túbulo, o útero, que se torna distendido com ovos nos helmintos grávidos (Figura 3). O útero também pode conter espermatozoides de uma copulação recente, os quais foram depositados na sua porção terminal e seguem migrando em direção ao receptáculo seminal. Na porção terminal do útero a parede é muscular servindo como uma câmara (metratermo) que recebe o órgão copulador masculino, localizado na bolsa do cirro (Souza et al., 2011) (Figura 4).

Figura 2 - Esquema da morfologia geral de *Echinostoma paraensei*
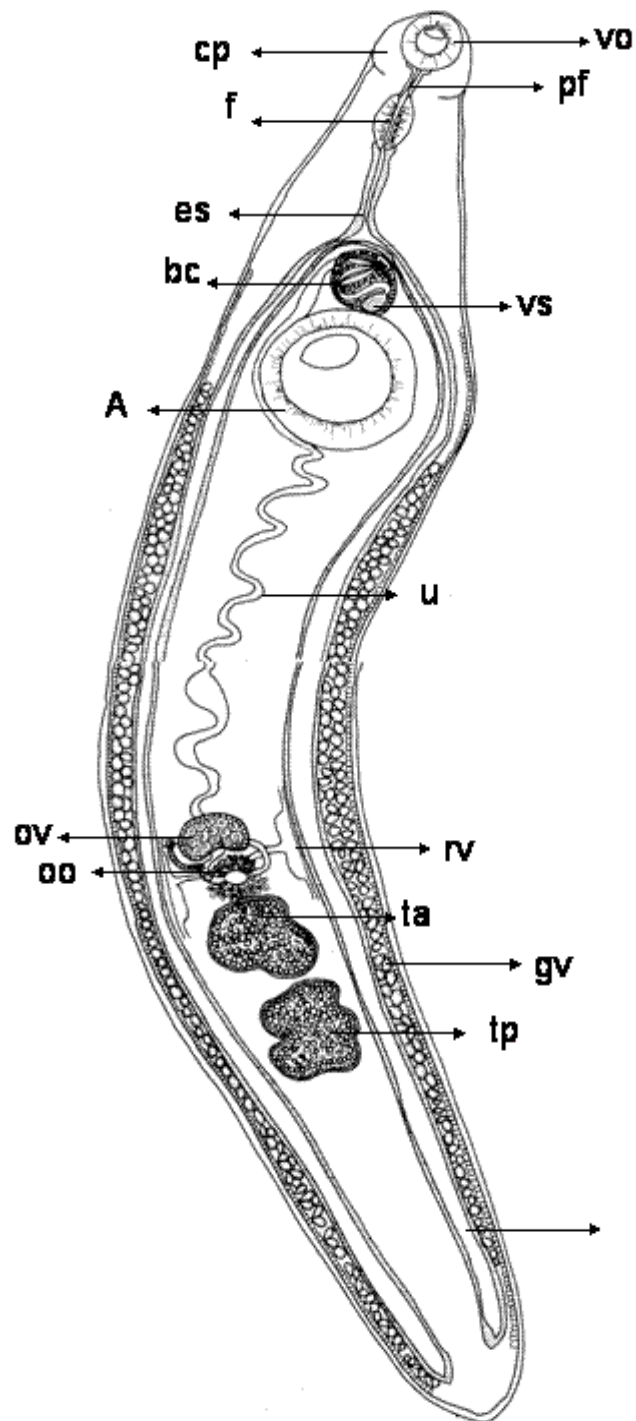
Legenda: Ventosa oral (vo), colar peristomático (cp), pré-faringe (pf), faringe (f), esôfago (es), bolsa do cirro (bc), vesícula seminal (vs), acetábulo (A), útero (u), ovário (ov), reservatório vitelínico (rv), oótipo (oo), testículo anterior (ta), glândulas vitelínicas (gv) e testículo posterior (tp). Esquema da autora.

Figura 3 - Esquema do sistema reprodutor de *Echinostoma paraensei* adulto (21 dias)
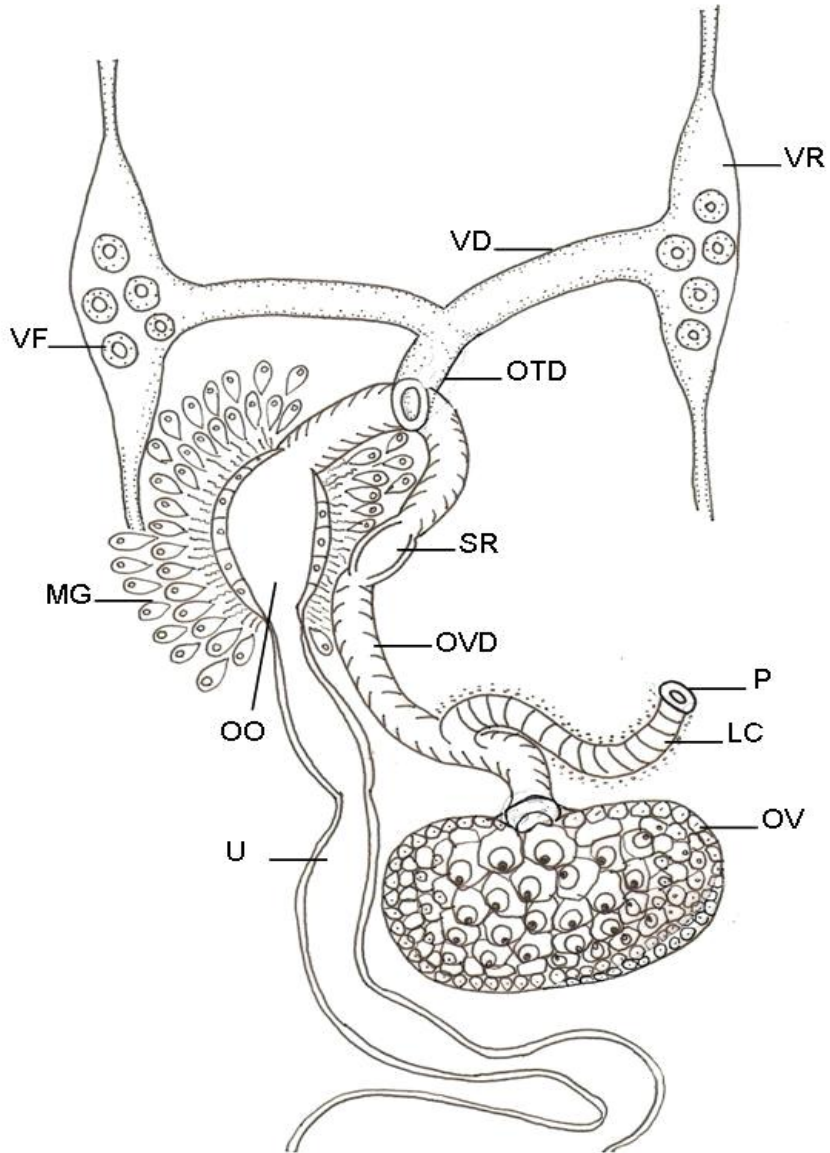
Legenda: Ovário (OV), poro (P) conectado com o canal de Laurer (LC), ovicduto (OVD), formando receptáculo seminal (SR), oótipo (OO), circundado pelas glândulas de Mehlis (MG), útero (U), reservatório vitelínico (VR) conectado com o ducto vitelínico (VD) que se juntam para formar o ducto ovovitelínico (OTD). Esquema da autora.

Figura 4 - Esquema do sistema reprodutor de *Echinostoma paraensei* adulto (21dias) mostrando a bolsa do cirro com o cirro introvertido
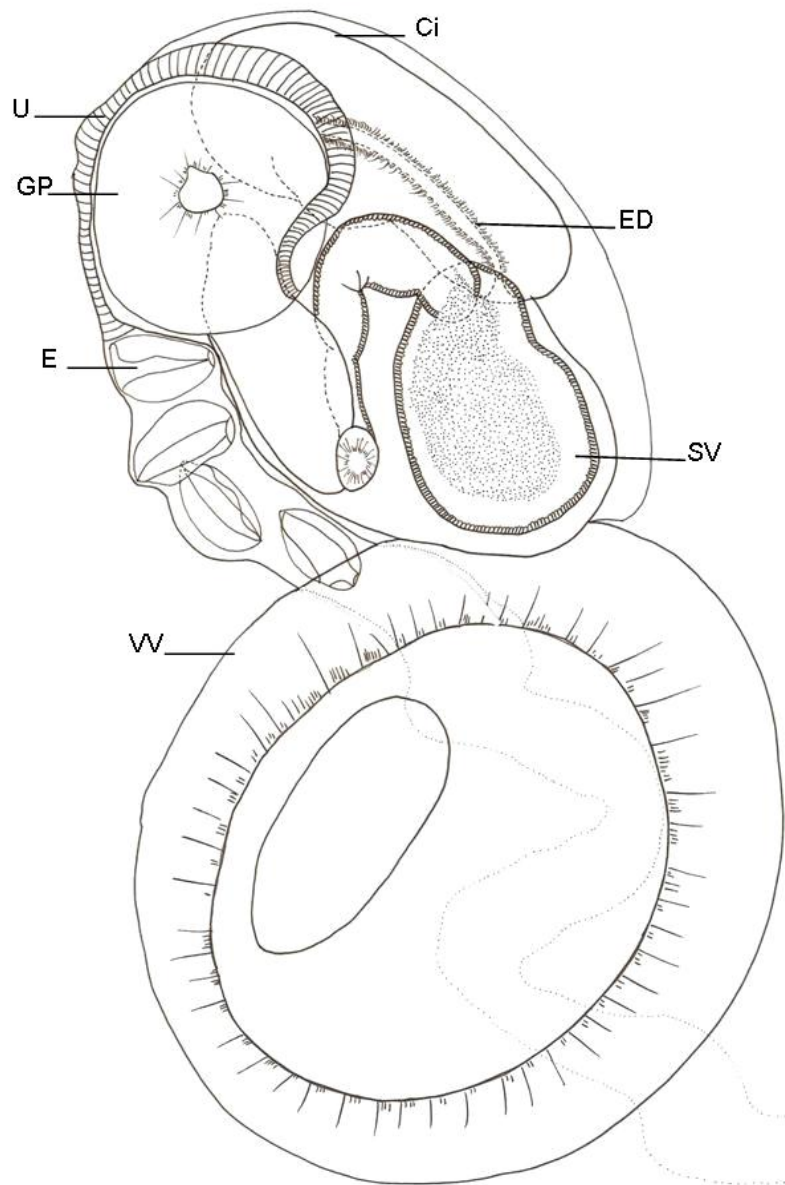
Legenda: Vesícula seminal [SV] conectado ao bulbo do ducto ejaculatório [BED]. Ducto ejaculatório [ED], cirro [Ci]. Utero [U] próximo à ventosa ventral [VV] carreando ovos [E] abrindo no metratermo ou gonoporo [GP]. Esquema da autora.

O sistema excretor é simples constituindo de um canal liso que segue da região posterior ao testículo posterior à extremidade posterior do corpo, terminando num poro denominado poro excretor (Maldonado et al., 2001a). Na metacercária recém desencistada é possível visualizar os túbulos excretores partindo da região anterior contendo concreções excretoras também chamadas de corpúsculos calcários (Saxton et al., 2008). As concreções excretoras são compostas por fosfato e carbonato de cálcio e magnésio e vestígios de uma variedade de elementos, tais como alumínio, cromo, ferro, manganês e cobre. A composição das concreções excretoras pode varia de acordo com a origem geográfica da espécie (Chalar et al., 2013).

Por meio da microscopia eletrônica de varredura é possível observar que os helmintos adultos (Figura 5) possuem as bordas laterais do corpo curvadas ventralmente no eixo longitudinal. No colar, os espinhos dorsais podem se retrair para uma bolsa formada pelo próprio tegumento. A ventosa oral apresenta três lábios. O colar de espinhos possui uma região de papilas sensoriais localizadas entre os espinhos do colar e os lábios da ventosa oral. O tegumento apresenta espinhos do tipo escamas que recobrem a superfície ventral da região anterior do corpo do helminto. O poro excretor localiza-se na porção subterminal e é recoberto por tegumento sem nenhuma estrutura característica (Maldonado et al., 2001a).

Figura 5 - Micrografias eletrônicas do helminto adulto de *Echinostoma paraensei.*
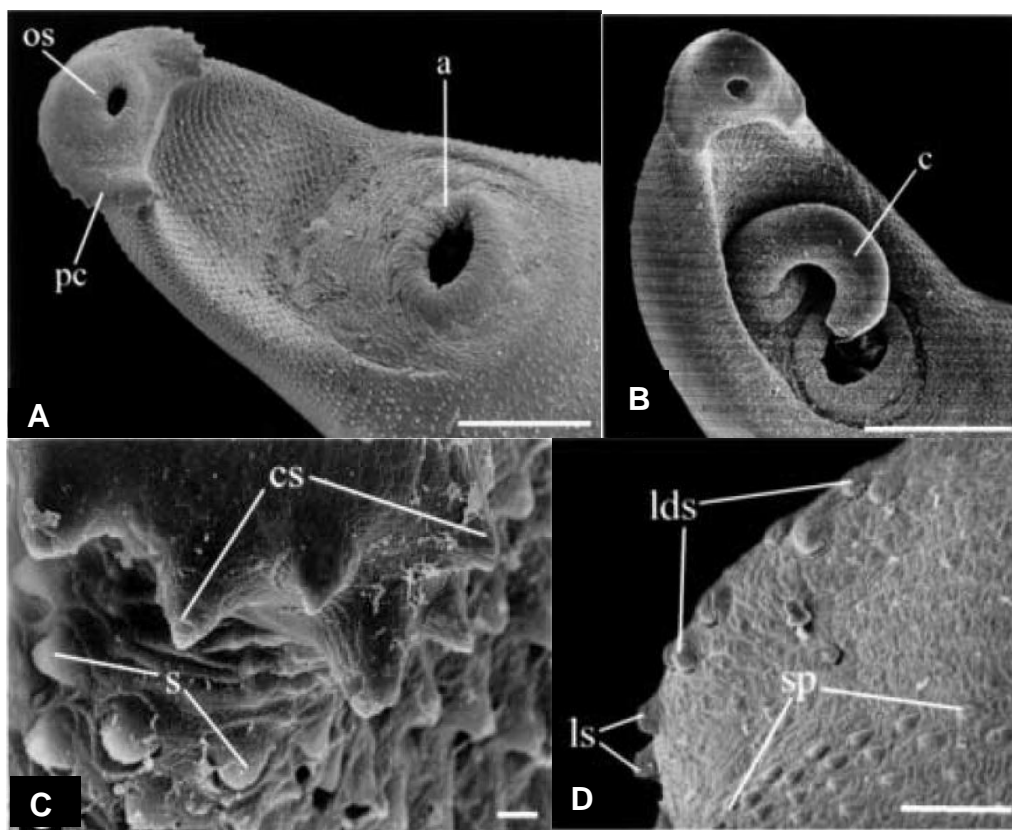
Legenda: (A) Vista ventral do helminto mostrando ventosa oral [os], colar de espinhos [pc] e o acetábulo [a]. Observe os espinhos do tegumento entre a ventosa oral e acetábulo. (B) vista ventral mostrando o cirro [c]. (C) Vista ventral mostrando o colar de espinhos e o cirro sem espinhos (c). (C) Espinhos do colar peristomárico [cs] e papilas sensoriais [s]. (D) Espinhos laterais [ls] e laterodorsais [lds] do colar de espinhos próximos às papilas sensoriais (sp).
Fonte: Maldonado et al., 2001a.

Ciclo biológico de *Echinostoma paraensei*

Os membros do gênero *Echinostoma* e gêneros relacionados apresentam um ciclo biológico com três hospedeiros: um vertebrado definitivo, um primeiro hospedeiro intermediário (usualmente um molusco gastrópode aquático), e um segundo hospedeiro intermediário, que abriga o estágio de metacercária encistada. O ciclo é realizado em seis fases, resumidamente: 1) Passagens de ovos imaturos pelas fezes do hospedeiro definitivo para o ambiente e seu subsequente desenvolvimento até a fase de ovo embrionado; 2) Eclosão do miracídio, contato e penetração no primeiro hospedeiro intermediário; 3) desenvolvimento e multiplicação no interior do caramujo; 4) Emergência de cercárias do caramujo e contato com o segundo hospedeiro intermediário; 5) Penetração e encistamento da cercária no

segundo hospedeiro intermediário e 6) Finalmente, ingestão de metacercárias pelo hospedeiro definitivo e desenvolvimento do helminto adulto no intestino (Fried e Grackzyc, 2004).

Os ovos de *E. paraensei* são encontrados nas fezes do hospedeiro definitivo aproximadamente aos 14 dias após a ingestão das metacercárias (Lie & Basch, 1967) (Figura 6). Os ovos mantidos em água destilada a 28 °C eclodem estimulados pela luz após 11 dias de incubação. Os miracídeos penetram ativamente pelas partes moles de *B. glabrata* desenvolvendo-se em esporocisto, que por reprodução assexuada dará origem às rédias. As primeiras rédias são observadas a partir do sexto dia de infecção e migram para vários tecidos do molusco (Lie & Basch, 1967). Cercárias são obtidas de rédias após o 25º dia de infecção e atingem a cavidade pericárdica do molusco, por meio do orifício urinário, rins e ducto ciliado renopericárdico. Seu encistamento pode ocorrer na região pericárdica de *B. glabrata* ou *B. straminea* (Lie & Basch, 1967) (Figura 6).

Os hospedeiros definitivos ingerem os moluscos infectados com as metacercárias de *E. paraensei*. Após a passagem pelo estômago, as metacercárias alcançam o intestino delgado, onde nas condições ideais de tensão de gás carbônico, temperatura, sais biliares e enzimas ocorre o desencistamento. Os helmintos se alojam no ducto pancreático e mais frequentemente na primeira porção do duodeno (Figura 6).

Figura 6 - Esquema do ciclo biológico de *Echinostoma paraensei*.
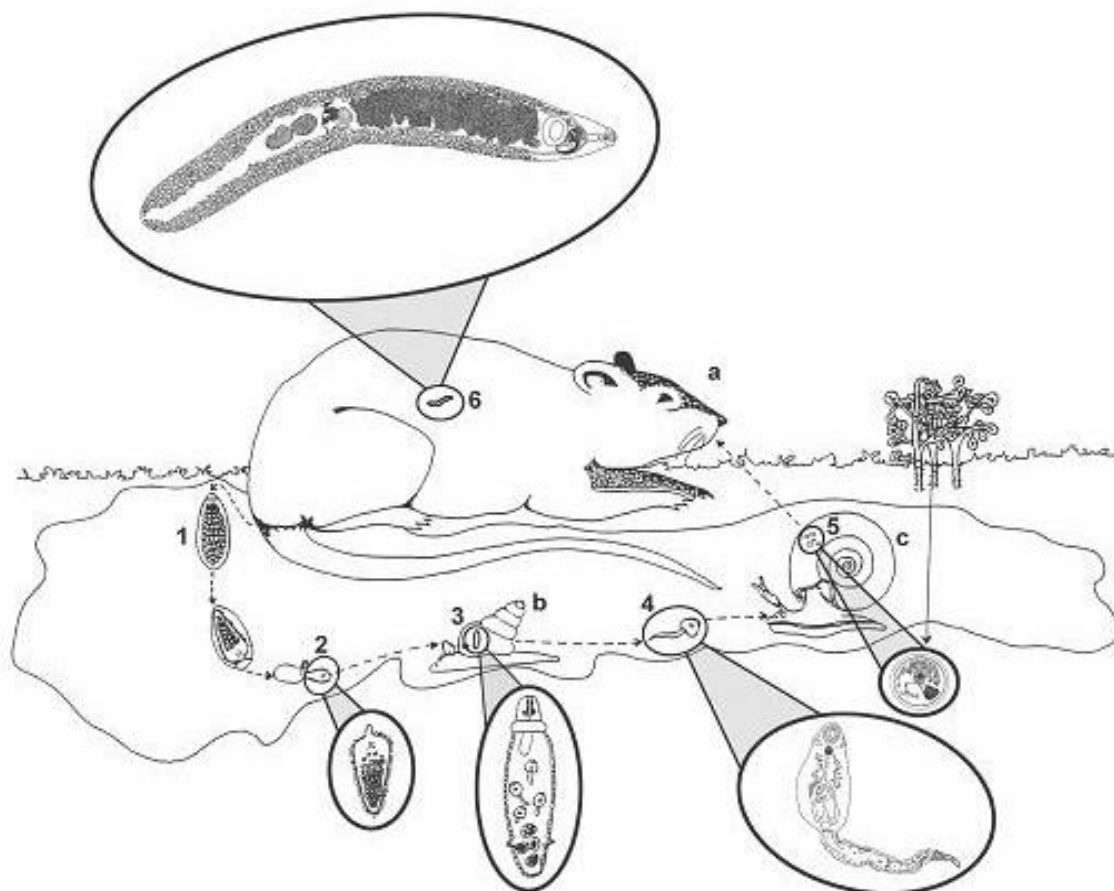
Legenda: (1) ovo operculado, (2) miracídio no ambiente, (3) rédia no interior do molusco, (4) cercária livre no ambiente, (5) metacercária no segundo hospedeiro intermediário (6) verme adulto no hospedeiro definitivo. (a) *Nectomys squamipes*, (b) *Lymnaea columella*; *Physa marmorata*, (c) *Biomphalaria glabrata* (Maldonado, 2002).

Equinostomíase, a doença.

Desde longo tempo, já é sabido que espécies de *Echinostoma* parasitam humanos. No Brasil, a sua presença foi demonstrada no período pré-colombiano pelos estudos paleoparasitológicos, que evidenciaram ovos de *Echinostoma* em coprólitos humanos mumificados similares a *Echinostoma paraensei* e *Echinostoma luisreyi* (Sianto et al., 2005; Leles et al., 2014).

Garrison (1908) assinalou o primeiro caso de equinostomíase humana nas Filipinas, pelo achado de ovos nas fezes de prisioneiros e 21 helmintos adultos recuperados de um paciente depois do tratamento. Os helmintos adultos foram identificados como *Echinostoma*

ilocanum. Depois disso, inúmeros registros de equinostomíase humana foram feitos na Ásia (Majima, 1927; Hirazawa, 1928; Anazawa, 1929).

Atualmente, a equinostomíase humana está atribuída a, no mínimo, 20 espécies de *Echinostoma*, que são endêmicas do sudeste da Ásia ao Extremo Oriente, cujos focos estão localizados na China, Índia, Indonésia, Coréia, Malásia, Filipinas, Rússia, Taiwan e Tailândia. Casos ocasionais também têm sido relatados em outros países (Grazczyk & Fried, 1998).

Nos países endêmicos a transmissão ocorre de forma focal estando associada a determinadas práticas socioeconômicas e culturais. A infecção ocorre pela ingestão de moluscos, peixes, crustáceos e anfíbios crus ou mal cozidos, nos quais as metacercárias são encontradas encistadas (Graczyk & Fried, 1998). Neste contexto, a equinostomatíase pode ser considerada uma zoonose transmitida por alimentos. No Brasil, não foram relatados casos de equinostomíase humana, entretanto por se tratar de uma doença veiculada por alimentos, existe a possibilidade de se tornar frequente futuramente, principalmente em países que vem incorporando novos hábitos alimentares, incluindo o consumo de pratos da cultura asiática, geralmente preparados com os moluscos, peixes, crustáceos e anfíbios crus.

A patologia da infecção representa um complexo conjunto de reações. A complexidade é dependente de uma grande variedade de fatores incluindo a espécie do parasito e as espécies de hospedeiros (Toledo et al., 2006). Doenças clínicas são moduladas pela intensidade da infecção e os principais sinais de infecção pesada são fraqueza, diarreia, emagrecimento, desconforto abdominal culminando em redução da capacidade de trabalho (Huffman & Fried, 1990; Toledo et al., 2006).

A morbidade e mortalidade devido à equinostomíase são difíceis de serem avaliadas em áreas endêmicas devido à prolongada fase de latência, fases assintomáticas, e também devido à similaridade de seus sintomas clínicos aos de outras helmintíases intestinais (Graczyk & Fried, 1998). Os sintomas de infecções leves a moderadas incluem anemia, diarreia, tonturas e dor abdominal, enquanto que as infecções severas estão associadas com diarreéia com grande perda de água, anemia, edema, anorexia, entre outros.

O fenômeno desencistamento

A maior parte dos digenéticos, exceto os do gênero *Schistosoma*, infecta o hospedeiro vertebrado quando os cistos de metacercárias ou mesmo o segundo hospedeiro vertebrado são

ingeridos por um hospedeiro definitivo (Maldonado & Lanfredi, 2009). Os helmintos jovens desencistam geralmente no intestino auxiliados por condições físico-químicas do hospedeiro definitivo (enzimas, temperatura, pH, sais de bile, tensão de CO_2, redutores e outros) e até mesmo do próprio parasito (secreções, atividade muscular e outros) (Fried, 1994).

Estudos têm demonstrado infecções por trematódeos e suas transmissões a humanos e animais, por meio de metacercárias encistadas por diferentes hospedeiros intermediários como peixes, crustáceos, insetos e moluscos, assim como a transmissão por plantas (Fried et al., 2004).

Metacercárias encistadas de *E. paraensei* podem ser obtidas de moluscos como *B. glabrata* naturalmente ou experimentalmente infectada, por meio da dissecação da região pericárdica, podendo ser estocadas em solução salina (Locker) a 4 ºC por até 6 meses ou, então serem utilizadas para infecções de hospedeiros definitivos ou desencistamento químico *in vitro (*Fried, 1994).

A morfologia das metacercárias varia de espécie para espécie nos digenéticos. Existe uma variação específica no número de camadas da parede do cisto e sua composição e por isso, diferentes metodologias têm sido testadas para o desencistamento *in vitro* em diferentes espécies (Fried, 1994). Em *E. paraensei*, os cistos formados na região pericárdica de *B. glabrata* possuem uma conspícua camada de fibras externa a uma camada denominada "outer cyst wall" seguida internamente por uma segunda camada denominada "inner cyst wall", esta última fica adjacente à superfície do helminto (Figura 7) (Stein & Basch, 1977). À medida que o desencistamento ocorre, os cistos de metacercárias alteram a sua morfologia.

Figura 7 - Fotomicrografia de campo claro de metacercárias de *Echinostoma paraensei*
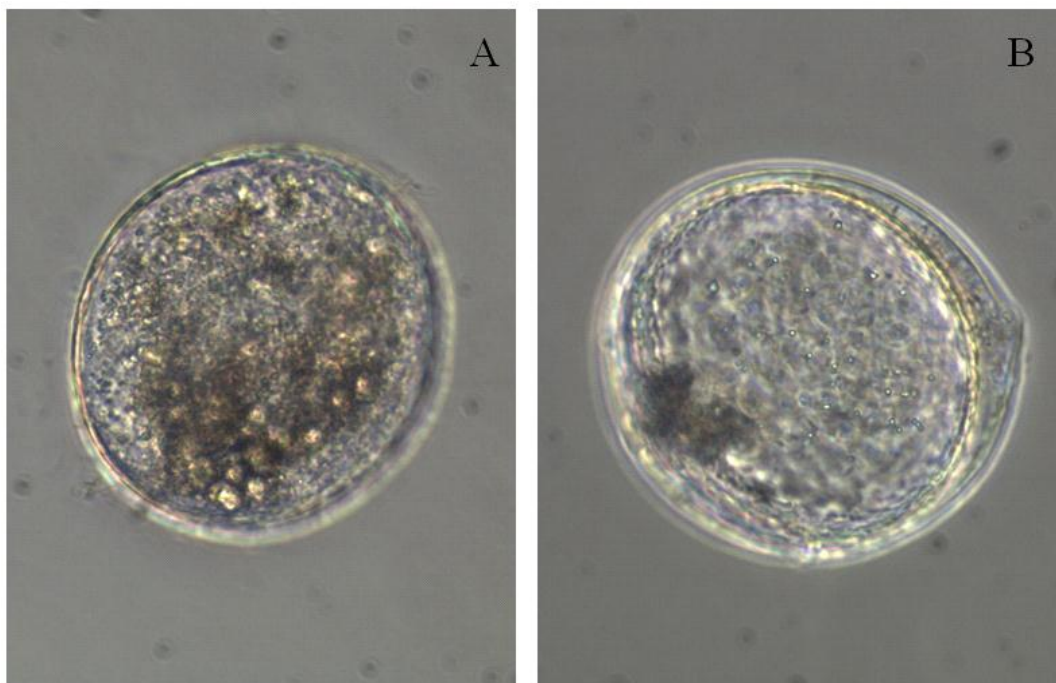
Legenda: (A) morfologia antes do desencistamento e (B) após a liberação da larva para o meio.

O desencistamento consiste na saída da metacercária do cisto auxiliada por combinações de fatores provenientes do microambiente do hospedeiro definitivo, e ainda fatores internos, relacionados à própria metacercária. Dentre os fatores do hospedeiro destacam-se a temperatura, o pH, a fase gasosa, as reações de oxidação-redução, a presença de enzimas, o pré tratamentos e os sais de bile. Por outro lado, a atividade motora da metacercária e a secreção de substâncias constituem fatores que podem auxiliar o desencistamento (Fried, 1994).

Em trematódeos digenéticos, metacercárias de diferentes espécies adotam diferentes fatores para realizar o desencistamento. Apesar de seus vários mecanismos, o desencistamento consiste, basicamente, na digestão da parede do cisto e sua ruptura pelos movimentos da larva (Ohyama, 1998). A parede interna do cisto é desgastada em um ponto e a metacercária sai por uma área fragmentada (Irwin & Fried, 1990). Há especulações de que secreções glandulares de helmintos possam estar envolvidas no rompimento da camada interna do cisto (Irwin & Fried, 1990). O desencistamento é completado quando a camada externa do cisto é rompida e a larva emerge.

Devido às variações no número de camadas do cisto das metacercárias e sua composição química, alguns métodos têm sido descritos para o desencistamento *in vitro* de

diferentes espécies associadas às características físico-químicas do hospedeiro e aquelas intrínsecas ao cisto.

Dos fatores físico-químicos advindos do hospedeiro definitivo que auxiliam o desencistamento, a temperatura parece ser um dos mais importantes para os digenéticos. Sabe-se que digenéticos de hospedeiros homeotérmicos, geralmente, desencistam num faixa de temperatura ótima entre 37 à 42 ℃, como por exemplo, *Parorchis acanthus* à 40 ℃ (Fried & Roth, 1974; Fried, 1994). Já as metacercárias de vertebrados poiquilotérmicos desencistam em temperaturas mais baixas, como por exemplo, espécies de *Plagiorchis* cuja temperatura ótima é 22 ℃ (Block, 1989). Os mecanismos pelas quais a temperatura influencia o desencistamento ainda não foram elucidados.

A maior parte dos estudos de desencistamentos é feito com a presença de uma fase gasosa. Existem evidencias que sugerem que o gás carbônico estimula o desencistamento. O bicarbonato de sódio ($NaHCO_3$) aumenta a infectividade de *Zygocotyle lunata*, quando administrado em aves e roedores, por diminuir a acidez do estômago ou aumentar o peristaltismo (Fried, 1970). O gás carbônico pode ser liberado e servir como estímulo para o desencistamento (Lackie, 1975).

Na família Echinostomatidae o desencistamento pode ocorrer dentro de uma faixa de pH entre 5,5 e 8,5 (Fried, 1994), embora haja exceções (Fried & Johnson, 2005).Alguns estudos tem demonstrado a influência do pH ótimo na saída da metacercária do cisto (Fried & Emili, 1988; Panic et al., 2013).

Alguns compostos, como ditionito de sódio e L-cysteína, apresentam potencial de oxi-redução e podem auxiliar o processo de desencistamento (Irwin et al., 1993). No entanto, os mecanismos pelas quais os redutores atuam nesse fenômeno não são claros. Os redutores são utilizados como um tratamento a parte do tratamento propriamente dito para o desencistamento. Geralmente, os redutores são utilizados entre o pré-tratamento (geralmente salina ácida ou pepsina ácida) e o tratamento com o meio para o desencistamento (tripsina alcalina ou tripsina-sais de bile alcalino). O tempo de exposição aos redutores é importante, pois longas exposições podem levar a larva à morte.

O desencistamento pode requerer a presença de enzimas como a pepsina e a tripsina (Wang & Statish, 1975). Para alguns digenéticos a pepsina ácida é o fator extrínseco mais importante (Fried, 1970). Outros digenéticos requerem a pepsina ácida como um pré-tratamento (Fried & Johnson, 2005). Para digenéticos de hospedeiros homeotérmicos não há requerimento obrigatório de pré-tratamento com pepsina ácida ou salina ácida e o

desencistamento ocorre na presença da enzima tripsina associada aos sais de bile (Panic et al., 2013). A tripsina é normalmente utilizada no desencistamento associada aos sais de bile ou tauracolato de sódio, atuando na digestão das camadas dos cistos, sendo esta enzima um fator extrínseco obrigatório para um ótimo desencistamento da maior parte dos digenéticos de homeotérmicos (Fried et al., 1994).

A bile é um complexo de substâncias incluindo a água, mucinas, proteínas, pigmentos, sais conjugados e não conjugados, lipídios, fosfolipídios e íons inorgânicos (Hasselwood, 1978). Estudos de desencistamento que associam bile à tripsina apresentam elevada taxa desse fenômeno, entretanto para alguns digenéticos o desencistamento pode ocorrer sem esses sais. Os sais de bile apresentam efeito sinérgico à tripsina e os mecanismos pelas quais atua ainda não são bem entendidos, não existindo um modelo simples para explicar a atuação dos sais de bile nas metacercárias (Fried, 1994).

Os fatores intrínsecos à metacercária que contribuem para o desencistamento incluem a secreção de substâncias e atividade motora, particularmente a muscular. Esse padrão comportamental durante o processo já foi visualizado em *Fasciola hepatica*, *Plagiorchis* sp.*, E. caproni* (Sukhdeo & Metrick, 1986; Bock, 1989; Panic et al., 2013). Estudos sugerem que a metacercária secrete enzimas durante o processo de desencistamento (Irwin et al, 1983; Fried & Stomberg, 1985; Chum et al., 1995), substâncias lipofílicas através do poro excretor (Johnston & Halton, 1981), lipídios neutros (Butler & Fried, 1977), grânulos (Gracyzk & Shiff, 1993), proteases que hidrolisam queratina (Hotez et al., 1993) e, por fim, liberam concreções excretoras (Smoluk & Fried, 1994; Fried & Reddy, 1997)

Desde a revisão bibliográfica feita por Fried (2000), poucos estudos têm usado o cultivo *in vitro* de metacercárias desencistadas do gênero *Echinostoma*.

Dos numerosos meios utilizados para promover o desencistamento químico, dois são usados com maior frequência. O primeiro meio foi descrito por Fried e Roth (1974) para desencistar *Parorchis acanthus* sendo constituído de tripsina-sais de bile alcalina preparada em solução salina balanceada de Earle (Earle's BSS), sendo chamado de meio TB (Trypsin Bile Salts medium). O outro meio foi descrito por Irwin et al. (1984) para estudos com *Himasthla leptosoma*é composto de uma mistura de salina alcalina contendo tripsina e sais de bile e redutor como a cisteína, referido como meio TBC (Trypsin Bile Salts Cysteine) (Saxton et al., 2008).

Como discutido por Fried (1994), o desencistamento químico é usado para obter metacercárias desencistadas com vários objetivos, tais como análise fenotípica por

microscopia de luz (ML),microscopia eletrônica de varredura (MEV), estudos sobre a fisiologia, bioquímica e imunologia.

Smoluk e Fried (1994) utilizaram a ML para obter novas informações no desencistamento de *Echinostoma trivolvis*. A metacercária foi examinada, seguida de desencistamento químico em meio TB, sendo estabelecidos os critérios de estágios de desenvolvimento. Os autores também determinaram os efeitos da manutenção dos cistos deste parasito em solução Locke 1:1 à 100 °C ou -20 °C e subsequentemente desencistados em meio TB.

O desencistamento químico foi usado por Ursone & Fried (1995) para obter novas informações durante o desencistamento de *E. caproni* por microscopia de luz. Especificamente, foram investigados os efeitos de vários meios e a manutenção dos cistos em solução Locke 1:1 a 100 e -20 °C.

Humphries & Fried (1996) realizaram estudos histológicos e histoquímicos de metacercárias desencistadas de *E. trivolvis* e *E. revolutum* obtidas seguindo o tratamento dos cistos em meio TB. Este estudo revelou a presença de glândulas com papel importante nos estágios de encistamento ou no desencistamento de *E. revolutum.*

Em 2000, Fried examinou as mudanças pós desencistamento em metacercárias de *Echinostoma caproni* e cultivou esse helminto em meio 199 enriquecido com 20% soro fetal bovino por sete dias a 41 °C em uma atmosfera de 5% de CO_2.

Poucos estudos foram publicados de 2000 a 2007, relatando o desencistamento químico *in vitro* de *Echinostoma* spp e seu cultivo. Nesse período, o efeito da estocagem de cistos de *E. caproni* em solução Locke 1:1 à 4 °C por 12 meses foi estudado por Rossi et al. (2001). Fried et al. (2004) desencistaram *E. caproni* e observaram os efeitos de diferentes nutrientes do meio de desencistamento nos cistos de metacercárias. Posteriormente, Fried & Peoples (2007a,b) estudaram a viabilidade de metacercárias encistadas versus metacercárias desencistadas, quimicamente, por microscopia de luz. Ao mesmo tempo, os autores estudaram os efeitos de vários fatores físicos e químicosna viabilidade de metacercárias encistadas. Apesar do desencistamento de algumas espécies do gênero *Echinostoma* ter sido relatado na literatura, poucos estudos têm demonstrado o cultivo e desenvolvimento *in vitro* desses helmintos. Esse fato demonstra o déficit existente a respeito do conhecimento científico sobre o desencistamento de metacercárias e cultivo de *E. paraensei*.

O desencistamento químico de *E. caproni* e *E. paraensei* foi feito por Fried e Reddy (1997) na tentativa de examinar, por microscopia de luz, as estruturas de metacercárias

desencistada, com o objetivo de determinar possíveis diferenças morfológicas entre essas espécies. Neste trabalho, o desencistamento de *E. paraensei* em meio contendo tripsina e sais de bile não foi obtido com sucesso.

De acordo com a literatura, as metacercárias de *E. caproni* são relativamente fáceis de serem desencistadas *in vitro* com uso do meio alcalino composto por tripsina e sais de bile (meio TB) à 41 ºC. A porcentagem de desencistamento dessa espécie no meio TB é geralmente maior que 90% (Rossi et al., 2001). Apenas um estudo tentou promover o desencistamento de *E. paraensei.* No entanto, apenas 13 % de larvas foram desencistadas (Fried & Reddy, 1997). O desencistamento químico é um bom indicador da viabilidade de cistos de *E. caproni*, uma vez que metacercárias desencistadas infectam camundongos (Rossi et al.,2001). Recentemente, Panic et al., (2013) investigaram o uso *in vitro* de larvas recém desencistadas de *E. caproni* para teste de drogas. A ação das drogas praziquantel, tribendimidine, albendazol e quinina foram avaliadas microscopicamente por 72 horas. Além disso, marcadores colorimétricos como resazurin, CellTiter-Glo® e Vybrant® foram testados como método alternativo de leitura. De acordo com esse estudo, as metacercárias permacem viáveis no meio de cultura no período de 5-7 dias. Os resultados da avaliação das drogas foram similares àqueles obtidos utilizando helmintos adultos em outros estudos e, por fim, os autores afirmaram que nenhum marcador pode ser usado com segurança, por não produzir sinais confiáveis ou citotoxicidade, sem grandes complicações.

Desenvolvimento de novas drogas com atividade antiparasitária

Aproximadamente 6000 espécies foram descritas na subclasse Digenea, entre elas 12 parasitam o homem. Nestas, estão incluídos helmintos de habitat sanguíneosangue (cinco espécies de *Schistosoma*), hepático (*Clonorchis sinensis*, *Fasciola gigantica*, *Fasciola hepatica*, *Opisthorchis felineus* e *Opisthorchis viverrim*), pulmonar (*Paragonimus* spp.) e intestinal (*Echinostoma* spp. e *Fasciolopsis buski*) (Keiser & Utzinger, 2009; Keiser, 2010).

Programas de controle de helmintíases na área de medicina humana (Smits, 2009) ou veterinária (Gavidia et al., 2010), em geral, são baseados no emprego de medicamentos. O controle terapêutico das helmintíases, incluindo as trematodíases de origem alimentar, é feito pela administração regular de fármacos anti-helmínticos, sendo o praziquantel ou o triclabendazol as atuais drogas de escolha (Keiser & Utzinger, 2004). O praziquantel tem um amplo espectro de atividade utilizado para clonorquiíase, opistorquíase, paragonimíase e infecções por helmintos intestinais (Keiser & Utzinger, 2004). Visto que o o praziquantel não é efetivo contra a fasciolíase, a alternativa é o uso do triclabendazol (Riper et al., 1990). A estratégia de controle medicamentosa pode apresentar resultados abaixo do esperado, devido à interferência de diversos fatores, entre os quais, a resistência do agente ao medicamento, o que tem trazido maior repercussão, principalmente na área da veterinária (Brennan et al., 2007). Em proporções ainda que menores, o tratamento da esquistossomíase tem chamado a atenção dos pesquisadores, que se preocupam com a resistência medicamentosa do praziquantel em algumas áreas endêmicas do mundo (Doenhoff et al., 2002). Uma das alternativas é o desenvolvimento de novos fármacos, que estão em fase de testes (Beckmann & Grevelding, 2010; Neves et al., 2010), ou mesmo produtos de origem vegetal (Araújo et al., 1991), que apresentem potencial (ou efeito comprovado) de atividade anti-helmíntica,uma vez que existe uma crescente preocupação sobre o desenvolvimento de resistência as duas drogas atualmente utilizadas para trematodíases, conforme acima referido . A resistência ao tricabendazol já é comum na parasitologia veterinária (Keiser et al., 2005). Além disso, o extensivo uso em massa do praziquantel em programas de administração de medicamentos aumenta ainda mais a preocupação sobre o surgimento de mais helmintos resistentes (Melman et al. 2009).

Apesar da ampla literatura abordando distintos aspectos da terapêutica experimental em diferentes trematodíases, os conhecimentos sobre este tema na infecção por espécies de *Echinostoma*, são bem mais restritos.

Artemisinina e seus derivados

A República Popular da China iniciou em 1967 um programa sistemático em busca de novos fármacos, empregando plantas nativas usadas como remédios em sua medicina tradicional, a fim de seguir em busca de novos fármacos que pudessem ter potencial antimalárico, uma vez que o número de casos desta parasitose tinha aumentado e tinham surgido novas cepas resistentes de *Plasmodium falciparum* (Klayman, 1985). Uma dessas plantas, *Artemisia annua L.*, já tinha uma longa história de uso. Conhecida como "qing hao" desde 168 a.C., foi usada inicialmente no tratamento de hemorroidas. No ano 340 d.C., ela foi descrita como antifebril no Manual de Prescrições para Emergências (Meshnick et al., 2002), e em 1798, a decocção de *A. annua* foi sugerida como tratamento para a malária (Klayman, 1985). Como resultado do programa iniciado em 1967, observou-se em 1971, que o extrato etéreo de *A. annua*, obtido à baixa temperatura, apresentava atividade antimalárica. Em 1972, foi isolado o princípio ativo, não relatado na literatura anteriormente. O princípio ativo da *A. annua* ficou conhecido no Ocidente como artemisinina (artemisinin) (Klayman, 1985). Em 1979, a estrutura da artemisinina foi determinada por difração de raios-X (Luo & Shen, 1987) e foi obtida sua síntese total no ano de 1983 (Schmid & Hofheinz, 1983).

Da sua fonte natural, *A. annua*, a artemisinina pode ser obtida por extração das folhas e flores (Frédérich et al., 2002). A planta é descrita como matéria prima na medicina chinesa para o tratamento de problemas no osso, entretanto os extratos já têm sido usados para o tratamento de febre, hemorroidas, piolhos e furúnculos.

A artemisinina é uma lactona sesquiterpênica trioxano que possui uma ligação endoperóxido. Desde seu isolamento, vários derivados da artemisinina foram sintetizados, visando melhorar a sua solubilidade, obtendo-se substâncias mais, ou menos ativas do que a artemisinina. Entre os derivados mais comuns, estão o diidroartemisinina (DQHS), arteméter, artemisiteno e artesunato (Figura 8), todos contendo o grupamento peróxido (Luo & Shen, 1987; Balint, 2001). Esses compostos foram denominados de endoperóxidos de primeira geração e são empregados na quimioterapia da malária na Tailândia, Vietnã, Brasil e China, onde a resistência ao parasito pode ser encontrada (Meshnick et al., 1996). A estrutura aparentemente complexa da artemisinina não é condição indispensável para a atividade antimalárica. Nos vários sistemas sintetizados e testados, o grupamento fundamental é a ligação endoperóxido (Meshnick et al., 1996).

Figura 8 - Estrutura química da artemisinina, artemeter, artesunato, arteéter, dihidroartemisinina e praziquantel
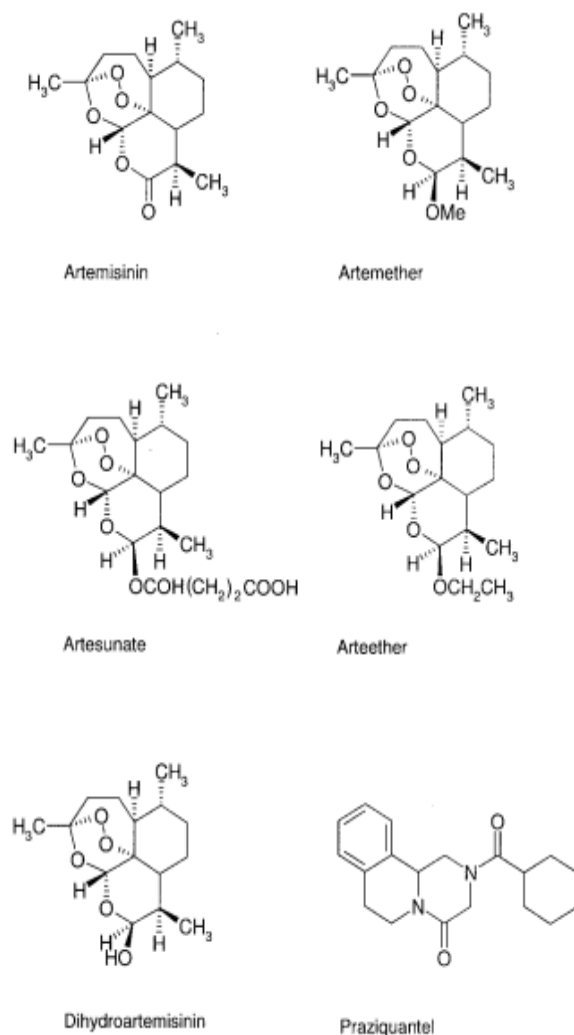
A fórmula empírica da artemisinina é $C_{15}H_{22}O_5$. Apresenta-se como um composto inodoro, incolor, podendo facilmente formar cristais com um ponto de fusão de 156-157 ° C. Sua massa molecular, como determinado pela espectrometria de massa, é m\z 282,1742. Essas propriedades permitem que os processos de extração e purificação sejam mais rápidos. Além disso, a artemisinina é relativamente barata, e facilmente disponível para as indústrias farmacêuticas, principalmente na Ásia (Dhingra, 1999; Haynes, 2001). Muitos derivados da artemisinina, denominados endoperóxidos de primeira geração, foram sintetizados e estão atualmente em uso ou sendo avaliados.

O uso de derivados da artemisinina é requerido não apenas devido ao rápido efeito e mecanismo de ação inovador, mas também em razão de sua aparente segurança. Nenhuma toxidade séria e poucos efeitos adversos foram observados em humanos (Dhingra, 2000).

Vários derivados de artemisinina apresentaram melhor solubilidade, estabilidade química e aumento da atividade antimalárica, sendo os mais importantes o arteméther, o artesunato e o arteether. Estudos têm relatado as propriedades medicinais, farmacológicas e terapêuticas deste novo grupo de antimaláricos (Klayman, 1985; de Vries & Dien, 1996; Ziffer et al., 1997; van Agtmael et al.,1999; Vroman et al., 1999; Dhingra et al., 2000; Price,2000). Ao longo dos últimos 20 anos, mais de dois milhões de pacientes que sofrem de malária foram tratados com derivados de artemisinina (Price et al., 1999). Vigilância clínica detalhada revelou que esses fármacos são seguros, não mostram efeitos adversos e zeram a parasitemia e sintomas relacionados com a malária, de forma mais rápida do que qualquer outro conhecido agente antimalárico já descoberto (Hien & White, 1993; WHO, 1998; McIntosh & Olliaro, 2000; Price, 2000).

No *Plasmodium*, a artemisinina tem o potencial de deformação, rompimento e degeneração das membranas do vacúolo digestivo e núcleo, mitocôndria, retículo endoplasmático e nucleoplasma, havendo a formação de vacúolo autofágico e perda de citoplasma levando o *P. falciparum* à morte num período de 8 à 24h em camundongos.

Os derivados arteméther e artesunato têm sido principalmente empregados como combinações terapêuticas para evitar ou retardar o surgimento de parasitos resistentes à droga, evitando o agravamento da doença. O uso racional da combinação de duas ou mais drogas com mecanismos independentes de ação, é uma tentativa de prevenir o surgimento de organismos mutantes resistentes emergentes. Enquanto a artemisinina causa uma redução substancial da carga parasitária do protozoário, os parasitos remanescentes são mortos por uma concentração considerável do fármaco.

Neste contexto, os derivados da artemisinina têm o seu potencial anti-helmíntico sido testado *in vitro* e *in vivo* para um grupo de helmintos causadores de trematodíases, sejam de habitat sanguíneo, teciduais ou intestinais. A principal motivação é a disponibilidade apenas dois fármacos praziquantel tricabendazol (Keiser & Utzinger, 2004) diante da reconhecida resistência a estes fármacos (Duthaler 2010; Duthaler et al., 2012).

A atividade esquistossomicida da artemisinina foi descoberta em 1980 por um grupo de cientistas chineses. O fármaco, administrado a vários animais infectados experimentalmente com *Schistosoma japonicum*, resultou em reduções marcantes da carga parasitária (Chen et

al., 1980). Em 1982, estas propriedades foram confirmadas para arteméther (Le et al., 1982). Ratos ou cães infectados com *S. japonicum* e tratados com arteméther em várias doses e vias de administração, mostraram alta redução das taxas de helmintos, variando entre 55 e 99% (Le et al., 1982). Os esquistossômulos (estágios larvais migratórios do parasito também foram susceptível ao arteméther (Le et al., 1982), mas nenhum efeito foi visto em ovos (Yue et al., 1984). Estudos subsequentes em laboratório confirmaram as propriedades esquistossomicidas do artesunato (Le et al., 1983), arteméther (Yin et al., 1991, Xiao et al, 1992) e, também para dihidroartemisinina (Abdel Aziz & el-Badawy, 2000).

Estudos com camundongos demonstraram que arteméther mostra maior atividade contra esquistossômulo de 14 a 21 dias de idade, com reduções na carga parasitária de 75-82%, entretanto é menos ativo em helmintos adultos (Xiao & Catto, 1989; Xiao et al., 2000b).

A superfície mais externa do tegumento dos trematódeos que interage com o hospedeiro é formada por duas camadas lipídicas justapostas, enquanto que a superfície basal interna é limitada por uma única membrana de camada dupla. O tegumento que é a interface entre o parasito e o hospedeiro, está envolvido na absorção de nutrientes, tem funções secretoras, protege os parasitos contra o sistema imunológico do hospedeiro e também é um importante alvo para drogas esquistossomicidas. O praziquantel é particularmente mais ativo contra helmintos adultos. É sabido que logo após 15 minutos de administração do praziquantel há alterações do tegumento, com formação de vesículas (Xiao et al., 1985; Shaw & Erasmus 1987; Gonçalves et al., 2013), seguido por vacuolização, rompimento dos tubérculos, levando à extensos danos no tegumento (Becker et al., 1980; Mehlhorn et al., 1981).

A destruição das ventosas oral e ventral, o inchaço e fusão de organelas tegumentares, descamação e erosão, desaparecimento de espinhos e papilas sensitivas são as alterações mais comuns no tegumento de helmintos causadas por derivados da artemisinina e observadas por microscopia eletrônica de varredura (Xiao et al., 1996b, 2000d, 2001b; 2003; Joraungkooorskul et al., 2006; Keiser & Morson, 2008; Tansatit et al., 2012). As principais alterações evidenciadas por microscopia eletrônica de transmissão são a formação de vacúolos, perda da definição da membrana basal, lise e desaparecimento da musculatura, severos danos às estruturas sensoriais e ao tecido parenquimal, diminuição do retículo endoplasmático granular e microvilosidades intestinais (Xiao et al., 1996a, 2001c; Tansatit et al., 2012). Após o tratamento, os danos tendem geralmente a diminuir gradualmente, enquanto os helmintos são capazes de mostrar sinais de recuperação parcial ou total (Xiao et al., 1996b, 2000d, 2001).

Uma das alterações bioquímicas estudadas *in vivo* em helmintos adultos tratados com derivados da artemisinina foi a redução do conteúdo de glicogênio no parasito. No período de 1 a 3 dias após o tratamento, o conteúdo de glicogênio foi reduzido em 28 -78% (You et al., 1994a; Xiao et al., 1997), parcialmente paralelo à diminuição da captação de glicose. No estudo constatou-se que o as reduções de glicogênio estavam mais relacionadas à inibição da glicólise do que uma interferência na absorção de glicose (Xiao et al., 1997). Outros estudos revelaram que *Schistosoma* spp. recuperado de camundongos tratados com arteméther apresentaram um aumento na atividade da enzima glicogênio fosforilase havendo um aumento significativo da fosforilase total na conformação ativa, enquanto que a conformação inativa não mostrou algum ou aumentos ligeiros (Xiao et al., 1999). Portanto, a redução do teor de glicogênio pode ser explicada pelo aumento na conformação fosforilase ativada, e em menor medida, a absorção de glicose diminuída (Xiao et al., 2000c).

Foram também observadas reduções nas atividades das enzimas fosfofrutoquinase, fosfoglicerato quinase, piruvato quinase, bem como na lactato desidrogenase de *S. japonicum* recuperados de camundongos tratados com derivados de artemisinina (Xiao et al., 1998a,b). No entanto, a redução das atividades dessas grandes enzimas da via glicolítica pode refletir em mudanças não-específicas que ocorrem concomitantes aos danos no parasito induzidos pela droga (Xiao et al., 2000c). Por fim, já foi relatado que o arteméther foi capaz de inibir ATPases, particularmente a Na+-; K+-APase e a Mg2+-ATPase (Xiao et al., 2000a,c).

Achados interessantes a respeito da ação de derivados da artemisina foram obtidos *in vitro* com *S. japonicum*, *S. mansoni* e *S. haematobium* adultos. Esses helmintos foram incubados em meio contendo arteméther (0.5-20 mg / mL) e hemina (50 -100 mg / mL). Pouco após a exposição, os helmintos apresentaram diminuição da atividade motora, seguido de um aumento gradual de vesículas do tegumento e morte do parasito, dentro de 24-72 h. Foi sugerido que a interação entre o arteméther e hemina produz um efeito tóxico sobre os helmintos (Xiao et al., 2001a). Curiosamente, este achado pode ser análogo ao que tem sido proposto a respeito do mecanismo de ação de derivados de artemisinina em *Plasmodium* sp. O mecanismo de ação envolve duas etapas sequenciais: 1) a ativação da artemisinina dentro do parasito pelo grupamento heme, que conduz à clivagem da ponte de endoperóxido e geração de radicais livres. Neste há interação dos derivados da artemisinina com grupamento heme gerado no vacúolo alimentar por digestão da hemoglobina, podendo gerar cisão da ponte de endoperóxido e 2) por conseguinte, a formação de ligações covalentes entre os radicais livres alquilados, e alquilação e oxidação de proteínas interagindo com centros ferro-enxofre de

enzimas e lipídios específicas para a malária que matam o parasito (Meshnick et al., 1996; Posner & Meshnick, 2001; Robert et al., 2005; Hartwing et al., 2009).

Outras propostas têm sido relatadas para tentar explicar os mecanismos de ação dos derivados da artemisinina, entre essas que esta droga pode estar interferindo com a função de flavoenzimas (Haynes et al., 2010). Outro estudo mostrou que a mitocôndria do parasito é um importante alvo direto (Li et al., 2005; Wang et al; 2010). Derivados de artemisinina podem interagir com a cadeia de transporte de elétrons do parasito gerando radicais livres, o que inibi a função mitocondrial, levando à morte do parasito.

Obtenção de material para estudos *in vivo* e *in vitro* com *Echinostoma*

A necessidade de fonte de material vivo e seguro é importante para pesquisa continuada com *Echinostoma* e equinostomíase. A situação ótima para a pesquisa continuada em *Echinostoma* é ter o ciclo biológico completo que se completa em curto espaço de tempo em laboratório (Figura 9). Portanto, a depender do objetivo, o material biológico podem ser formas larvárias, que são isoladas do molusco *B. glabrata*, Caso o interese seja dispor de equinostomatídeos adultos, vários animais de laboratório tais como hamsters, camundongos, ratos e galinhas podem ser infectados. Frequentemente, os helmintos adultos tem sido a mais disponível para pesquisa e alguns estudos no Brasil (Ferraz et al., 2012; Gonçalves et al., 2013).

Figura 9 - Representação do ciclo biológico de um *Echinostoma* mostrando os pontos de coleta de material biológico
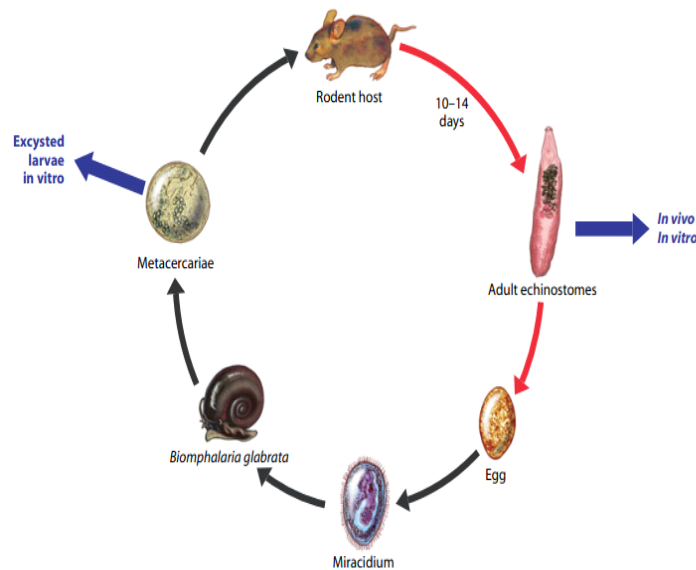
Fonte: Keiser et al.,2010.

O estudo *in vitro* de larvas recém desencistadas obtidas através do desencistamento químico permite a obtenção em grande número de helmintos em curto espaço de tempo, além de ser barata. Além disso, é um modelo que atende à política dos princípios dos 3R's reforçada ultimamente: refinar, reciclar e substituir (refine, recicle e replace. Ao mesmo tempo é uma fonte de material biológico, vivo e fresco, que pode, principalmente, ser utilizado para a avaliação de efeitos de produtos, substâncias, componentes, fármacos que ofereçam potencial anti-helmíntico. O modelo ainda substitui o sacrifício do hospedeiro definitivo (roedor) em fase preliminar de teste. Utiliza as metacercárias encistadas que podem ser consideradas fontes de material biológico em "dormência", facilmente estocadas, prontas para serem utilizadas.

Larvas desencistadas podem ser mantidas em *in vitro* em meio de cultura podendo ser útil para estudos biológicos, fisiológicos e terapêuticos. Alguns estudos têm analisado *in vitro* as atividades trematocidas de fármacos, utilizando trematódeos adultos, inclusive *Echinostoma* (Keiser et al., 2006; Panic et al., 2013). Neste caso, os helmintos incubados na presença da droga são analisados quanto à ocorrência de morte (mortalidade), motilidade e alterações morfológicas.

A relação parasito/hospedeiro no modelo *Echinostoma paraensei*

Embora espécies do gênero *Echinostoma* tenham ampla variedade de hospedeiros definitivos, é sabido que existe um maior grau de compatibilidade entre espécies de roedores. Entretanto, já foi documentado que algumas espécies apresentam um nível maior de compatibilidade com hamster e camundongo (Hansen et al., 1991; Mahler et al., 1995; Toledo et al., 2003). No entanto, o rato revela um menor grau de compatibilidade. Essa classificação é baseada em estudos que levam em consideração o estabelecimento e sobrevivência desses trematódeos em cada hospedeiro. Por exemplo, *E. caproni*, pode sobreviver mais que 20 semanas em camundongos e hamsters. Os hospedeiros mais compatíveis desenvolvem uma capacidade mais limitada de expelir/expulsar o helminto resultando em infecções de longa duração. No laboratório, hamsters são mais comumente usados para estudos com *Echinostoma in vivo*, uma vez que esse modelo permite um rápido período de infecção patente (10-14 dias) quando comparado a *Schistosoma* sp. e *Fasciola sp.*.

Toledo et al. (2004) avaliaram o desenvolvimento de *E caproni* em diferentes hospedeiro, a fim de determinar variabilidades específicas neste helminto adulto provocadas pela espécie de hospedeiro. Foi levada em consideração a taxa de liberação de ovos, taxa de recuperação e variáveis morfométricas dos helmintos. Os achados deste estudo revelaram que o modelo *E. caproni*/hamster exibiu um maior nível de compatibilidade, caracterizado por uma infecção de longa duração, grande liberação de ovos e taxas de infecção associados com um grande crescimento dos helmintos, quando comparados ao modelo *E.caproni*/rato (Toledo et al. 2004a,b).

De acordo com Toledo et al. (2004b, 2005) a infecção por *E. caproni* apresenta efeitos patológicos específicos em seus hospedeiros definitivos, hamster e rato. Em rato, foram observadas destruições significantes e erosão das vilosidades intestinais acrescida de notável aumento de células caliciformes ("goblet cells"). Já em hamster, as destruições e erosão das vilosidades foram observadas, mas acrescidas de infiltrados celulares (linfócitos, macrófagos e mastócitos) no mesentério, associado ao aumento de neutrófilos na mucosa, indicando inflamação local. Desta forma, ocorrem diferenças locais na resposta inflamatória entre esses hospedeiros em relação a *E. caproni*, uma vez que foi detectada marcada infiltração celular inflamatória e aumento do número de neutrófilos na mucosa em hamsters mas não em ratos.

Apesar de ter sido elucidado que espécies do gênero *Echinostoma* podem ter desenvolvimentos diferentes de acordo com a espécie de hospedeiro, pouca tentativa tem sido feita para avaliar os efeitos na morfologia dos helmintos jovens a adultos.Alterações morfológicas foram evidenciadas quando helmintos adultos eram mantidos em microambientes desfavoráveis. Os helmintos adultos com retração e perda de espinhos no colar foram assinalados em *E. trivolvis* (Kruse et al., 1992), somente retração em *E. caproni* (Fujino et al., 1994) e mudanças nos espinhos das ventosas (Sotillo et al., 2010).

1 OBJETIVOS

1.1 Objetivo Geral

Acompanhar a cinética de desenvolvimento e crescimento de *Echinostoma paraensei* em hospedeiros com diferentes graus de permissividade. Avaliar as condições de desencistamento e a aplicação do cultivo *in vitro* de formas jovens de *E. paraensei* como modelo para teste de fármacos de origem vegetal como o artesunato, que tem efeito antiparasitário.

1.2 Objetivos específicos

a) Avaliar o grau de compatibilidade entre *E. paraensei*, hamster e rato Wistar pela análise morfométrica e pela morfologia do helminto adulto, por microscopia de campo claro e microscopia laser confocal;

b) Avaliar se o grau de permissividade do hospedeiro interfere nas características alométricas do desenvolvimento e crescimento em *E. paraensei*.;

c) Caracterizar as fases do desenvolvimento de metacercárias de *E. paraensei* após o desencistamento químico *in vitro*;

d) Descrever as características morfométricas e morfológicas de helmintos jovens desencistados *in vitro* por microscopia de campo claro e microscopia de varredura laser confocal;

e) Investigar a atividade *in vitro* trematocida do artesunato, analisando as alterações morfológicas induzidas por esse fármaco em *E. paraensei* jovem recém desencistado, pelas microscopias de campo claro e microscopia eletrônica de varredura;

2 **PATTERN OF GROWTH AND DEVELOPMENT OF *ECHINOSTOMA PARAENSEI* (TREMATODA: ECHINOSTOMATIDAE) IN HAMSTER AND WISTAR RAT USING LIGHT AND CONFOCAL LASER SCANNING MICROSCOPY (ARTIGO CIENTÍFICO SUBMETIDO)**

Pattern of growth and development of *Echinostoma paraensei* (Digenea: Echinostomatidae) in hamster and Wistar rat using light and confocal laser scanning microscopy.

Joyce G. R. Souza[a], Juberlan S. Garcia[b], Arnaldo Maldonado Jr.[b], Rosângela Rodrigues-Silva[c], José Roberto Machado-Silva [a*1].

[a] Laboratório de Helmintologia Romero Lascasas Porto. Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas. Centro Biomédico. Av. Professor Manoel de Abreu, 444/ 5º andar, Vila Isabel, 20511-070, Rio de Janeiro, Brazil.

[b] Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz. Av. Brasil 4365, Manguinhos, 21040-360, Rio de Janeiro, Brazil.

[c] Laboratório de Helmintos Parasitos de Vertebrados, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz. Av. Brasil 4365, Manguinhos, 21040-360, Rio de Janeiro, Brazil.

[*] **Corresponding author**

[1] JR Machado has a fellowship from the National Council for Scientific and Techonological Development (CNPq).

Keywords: *Echinostoma paraensei*, morphology, morphometry, light microscopy, confocal microscopy, golden hamster, Wistar rat.

Abstract

Echinostoma paraensei (Digenea: Echinostomatidae) lives in the duodenum and bile duct of rodents and is reported as a useful model for studies on the biology of flatworms. Here, we compared the growth and development of worms collected 3, 7, 14 and 21 days pos infection

from experimentally infected hamster (permissive host) and Wistar rat (less permissive hosts). Linear measurements and ratios were examined by light (morphology and morphmetry) or confocal microscopy. At day 3, either worm from hamsters or rats were small with poorly developed gonads. At seven day, worms increased in size and morphometric differences between hosts are statistically significant after this time. In addition, older worms (14 and 21 days of age) harvested from hamster showed normal gonads and vitelline glands laterally distributed on the body, whereas worms from rat showed atrophied reproductive system characterized by undeveloped vitelline glands and stunted ovary. The worm recovery in rat decreased from 29.3% (day 7) to 20.6% (day 14) and 8% (day 21), whilst it remained around 37% in hamster. In conclusion, to the best of our knowledge, this is the first appointment demonstrating that low permissiveness influences the reproductive system of echinostome since the immature stages of development. The phenotypic analysis evidenced that hamster provides a more favorable microenvironment for gonads development than rat, confirming golden hamster as a permissive host, whereas Wistar rat is less permissive host.

1. Introduction

The assessment of biodiversity of *Echinostoma* (Digenea: Echinostomatidae) is historically provided from naturally-infected snail host, surveyed mammals from the field or birds, including those with migratory behavior (   ). According to the main zoological taxonomic index in Europe (Fauna Europaea) 153 species have been supervised by Gibson (Gibson et al., 2014). Recent studies based on morphological and molecular tools clarified the existing controversies about the taxonomy and nomenclature of European species (Georgieva et al., 2014; Faltýnková et al., 2015). In addition to their high biodiversity, some species are of zoonotic concern for human health (Fried and Graczyk, 2004a).

Most of the information currently available about the outcome of *Echinostoma* sp. infection is derived from experimental studies conducted in rodents. Earlier efforts have focused on evaluating of *Echinostoma caproni* as experimental model, including different species of rodent. The results obtained indicated that both parasite age and host species determine the outcome of infection. Considering this situation, some species has been recognized as highly susceptive hosts, whereas other have low susceptibility (Hansen et al., 1991; Toledo el al., 2004a,b). Considering that echinostomes are easy to maintain and convenient for manipulation under laboratory conditions, several studies highlight this fluke as an extraordinary experimental model (Toledo and Fried, 2005).

E. paraensei was first described from naturally infected snails *Biomphalaria glabrata* from Brazil (Lie and Basch, 1967). However, the natural definitive host of this fluke has not been reported in Brazil until the 2000s. The first report of *E. paraensei* was in *Nectomys squamipes* (Rodentia: Sigmondontinae) from secluded Atlantic Forest fragments (Maldonado et al., 2001a). Subsequently, the fluke was recovered from both small intestine and bile ducts of wild rodents (Maldonado et al., 2001a).

During the past decades *E. paraensei*-infected rodents have played a crucial role for the understating of the host-parasite relationship, response to experimental therapeutic and alternative control (Fried and Reddy, 1997; Fujino et al., 2000; Maldonado et al., 2001a,b; 2005; Toledo et al., 2004,a,b; Pinheiro et al., 2005; Garcia et al., 2010, Ferraz et al., 2012; Gonçalves et al., 2013; Bonfim et al., 2014).

Experimental infection were first carried out, indicating that more susceptible host carry the infection for a longer period, in contrast to other less susceptible, in which the infections is limited (Mahler et al., 1995; Toledo et al., 2004a,b; Mahler et al., 2005; Toledo et al., 2006). Thus, golden hamster and mice are termed permissive hosts, in contrast to Wistar rat, a less compatible host (Muñoz-Antoli et al., 2014).

The morphology of adult worms is generally well matched to their environment and has been used as morphological criteria of parasite-host compatibility. Adult *E. caproni* grown in rats show lower parasite development (body area, suckers area, collar width, pre-pharynx and esophagus length, cirrus sac, ovary and testis area), indicating low compatibility compared to hamster (Toledo et al., 2004a). However, the susceptibility of laboratory hosts to *E. paraensei* based on morphological criteria remains less known (Maldonado et al., 2005; Garcia et al., 2011; 2012).

This paper explores the morphology of adult worms grown in hamster and Wistar rat based on light microscopy and the potential of confocal scanning electron microscopy to detail the anatomy of specimens. The mains advantages of this non-destructive technology are allowing examination of internal features without the need for dissection (Souza et al., 2011, Souza et al., 2013; Lelis et al., 2014) and the same specimen can be observed either by light and confocal microscopy (Machado-Silva et al., 1998). In addition, this technique is more rapid and cheaper than scanning electron microscopy (Neves et al., 2005). Finally, the knowledge obtained in this study will be useful for a better understanding of the role of animal experimental model for *E. paraensei*.

2. Material and methods

2.1. Ethics statement

Experimental animal protocols complied with the ethical procedures for animal experiments (Ellery, 1985) were approved by the Commission of Ethics in the Use of Animals from Fundação Oswaldo Cruz (permit CEUA L-074/08).

2.2. Parasite maintenance

A Brazilian isolate (Sumidouro, Rio de Janeiro) of *E. paraensei* was maintained in *Biomphalaria glabrata* snail and golden hamster (*Mesocricetus auratus*) at the Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios IOC-Fiocruz (Rio de Janeiro,

(Brazil), as described elsewhere Garcia et al. (2011). Briefly, golden hamster was infected by gavage to metacercariae derived from experimentally infected *B. glabrata* snails. In this study, encysted metacercariae of *E. paraensei* were removed from the pericardial cavity-kidney region of experimentally infected *B. glabrata*. Two-week-old outbred females golden hamsters and albino rat (*Rattus norvegicus*) were obtained from the Animal Breeding Center of Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil). Animals were housed in polypropylene boxes (40 × 33 cm) with stainless steel screen covers. Water and food (Nuvilab CR1, Colombo, Paraná, Brazil) were provided ad libitum.

2.3. Experimental design

Golden hamsters (n= 10) and white rat (n= 10) were each orally fed 100 metacercariae (Maldonado et al., 2001b). Two hamsters and two rats were euthanatized in a CO_2 camera on days 3, 7, 14 and 21 post-infection (pi). Worms were removed from the small intestine and rinsed briefly in Locke's solution to remove debris and then were fixed in AFA solution (2% acetic acid, 3% formaldehyde, 95% and 70° ethanol) under the slight pressure of a cover slip at room temperature. Worms were stained with hydrochloric carmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted in Canada balsam (Souza et al., 2011).

2.4. Morphometric and morphological study

Locations for measurements are seen (Figure 1), as proposed by Valero et al. (2001). Measurements were taken using draws with aid of a lucida camera. Measurements were made of either linear characters or ratios. Linear: body length (BL), body width (BW), collar spines length (CL), collar spine width (CW), oral sucker length (OSL), oral sucker width (OSW), pharynx length (PL), pharynx width (PW), ventral sucker length (VSL), ventral sucker width (VSW), anterior testes length (ATL), anterior testes width (ATW), posterior testes length (PTL), posterior testes width (PTW), ovary length (OL), ovary width (OW), distance between

anterior end of body to ventral sucker (A-VS), distance between suckers (OS-VS) and distance between posterior end of body to ventral sucker (P-VS). Ratios: oral sucker/ventral sucker (OS/VS), body length/ body width (BL/BW), body length/ Distance between posterior end of body to ventral sucker (BL/P-VS).

Spines collar, suckers, pharynx, esophagus, ceca, ovary, uterus, ootype, vitelline glands, testes, cirrus sac and ducts were analyzed by light microscopy (Axioscop 2 plus) and confocal laser scanning microscopy (LSM- 510 META Zeiss), using a 543 nm He/Ne laser and a LP 570 filter under reflected mode. Line drawings were made with the aid of a drawing tube.

Figure 1- Standardized measurements used in *Echinostoma paraensei*. Adapted to Valero et al 2001.
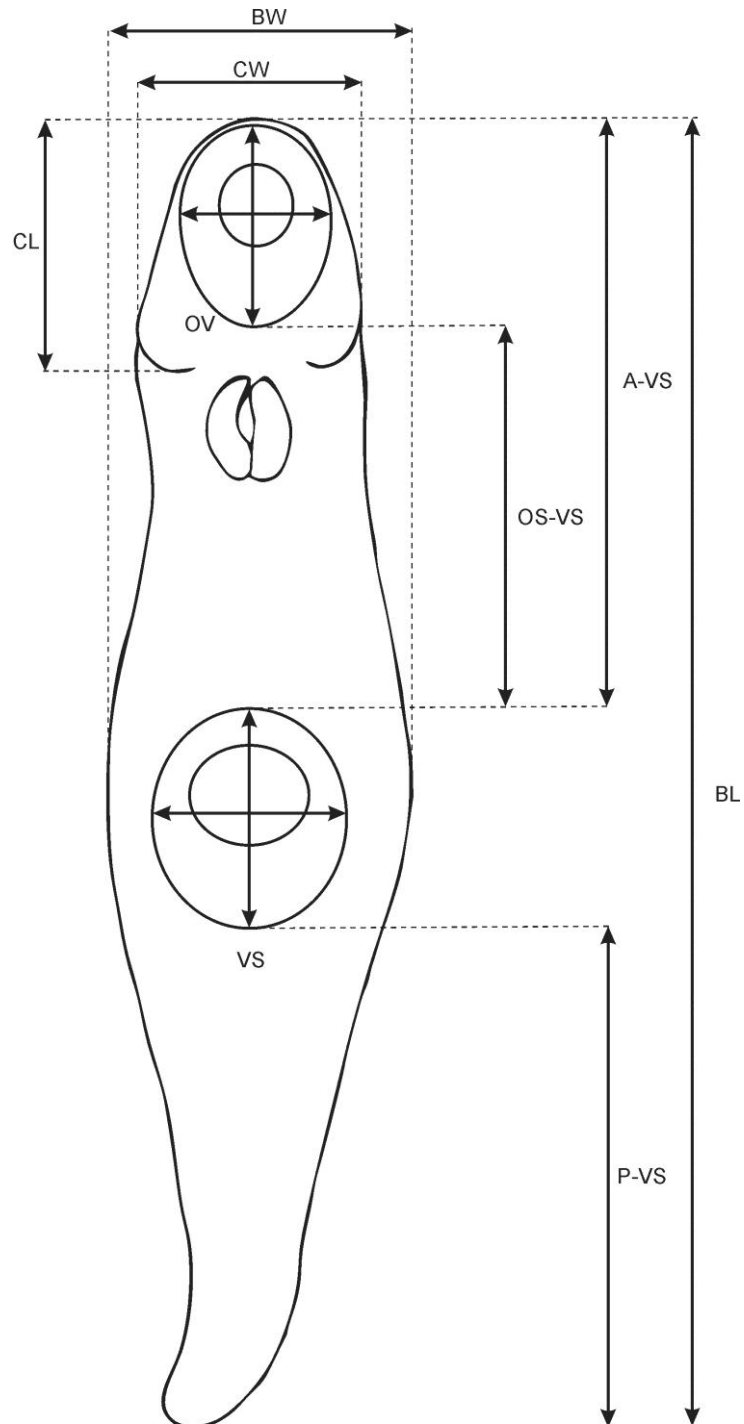
2.5. Statistical analysis

Quantitative data was analyzed using one-way ANOVA to compare means of morphological parameters of *E. paraensei* during developmental in each one host. After this, the Student's t-test was used to compare means of morphological characters of *E. paraensei* between hamsters and rat. The $p \geq 0.05$ was considered as statistically significant.

3. Results

3.1 Comparison of the morphometric data between hamster and rat

At any time analyzed, worm recovery was higher in hamster than rat: 15% and 12% (day 3), 37% and 29.3% (day 7), 36% and 20.6% (day 14) 37% and 8% (day 21). At this time interval, attachment sites (oral and ventral sucker and spine collar) and digestive system (pharynx, esophagus and caecum) were most evidenced structures by light microscopy.

The pattern of development is shown in figures 2, 3 and 4. Hamsters presented significantly higher body length mean than rat in all periods of infection analyzed. After seven days of infection there was a significant development of the body length (Figure 2), anterior end of body to ventral sucker region (A-VS), region between suckers (OS-VS) and posterior end of body to ventral sucker region (P-VS) (Figure 3). Three-old worms did not show differences regard the rate of development between the anterior and posterior end to ventral sucker (A-VS and P-VS) between hamster and rat. However, this rate differed in 7, 14 and 21-days old worms (Figure 3). Whereas the posterior end grew with high rate the anterior end did not follow this pattern with less intense increase (Figure 3).

Figure 2 - Comparative development of body length *Echinostoma paraensei* from hamster and Wistar rat 3 to 21 days post-infection.
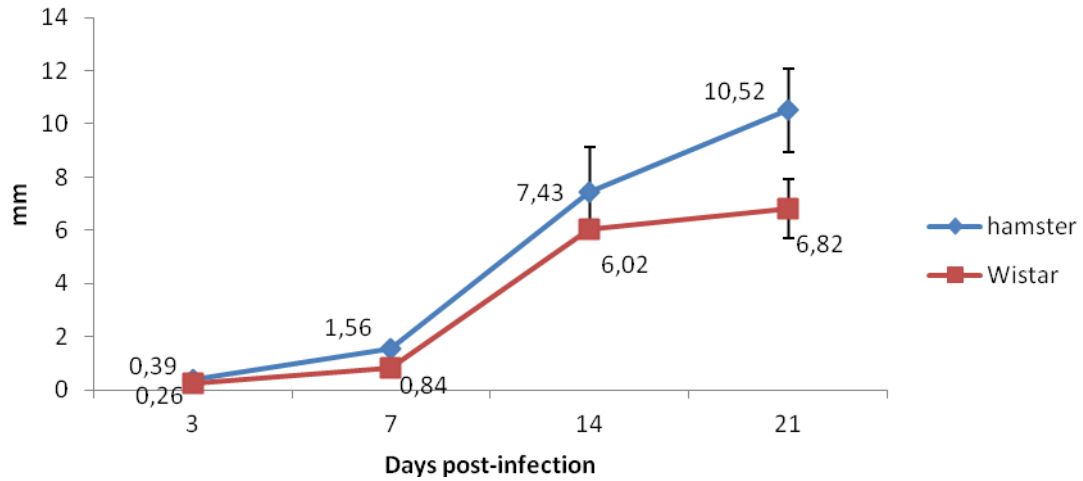
Figure 3- Comparative developmental of distance between anterior and posterior ends to ventral sucker of *Echinostoma paraensei* from hamster and Wistar rat between 3 to 21 days post-infection.
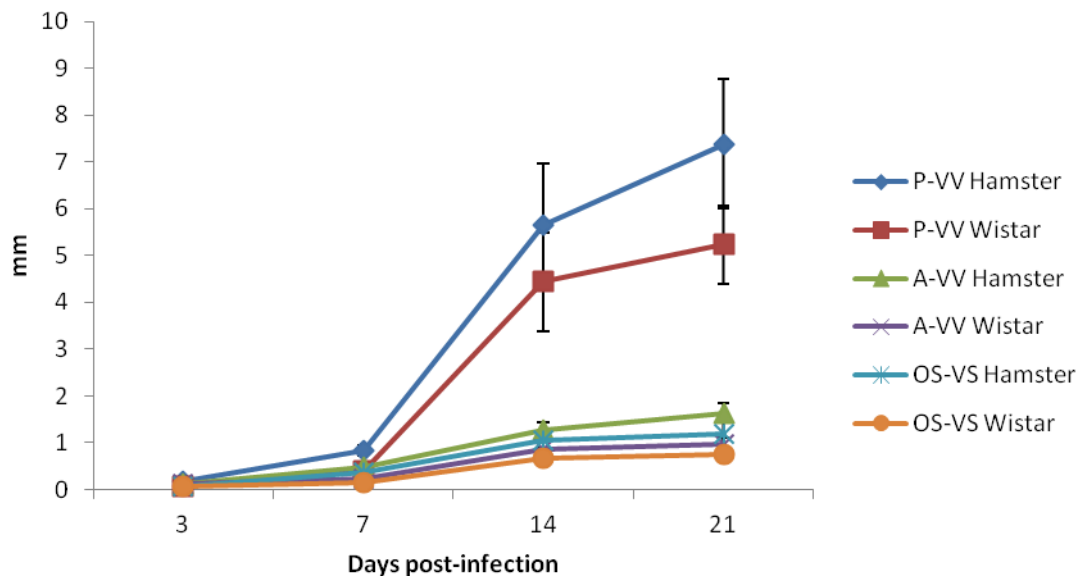
The body length/body width ratio (BL/BW), relation between suckers ratio (OS/VS) and body length /distance between posterior end of body to ventral sucker ratio (BL/P-VS) not showed any significant statistical difference during developmental in hamster or in rat when it was analyzed day-to-day. However, BL/BW ratio was significantly higher in rat than in hamster on 3 days post infection, but OS/VS ratio and BL/P-VS ratio showed similar values with no significant differences (Figure 4).

Figure 4 - Comparative ratio structures of *Echinostoma paraensei* from hamster and Wistar rat between 3 to 21 days post-infection.
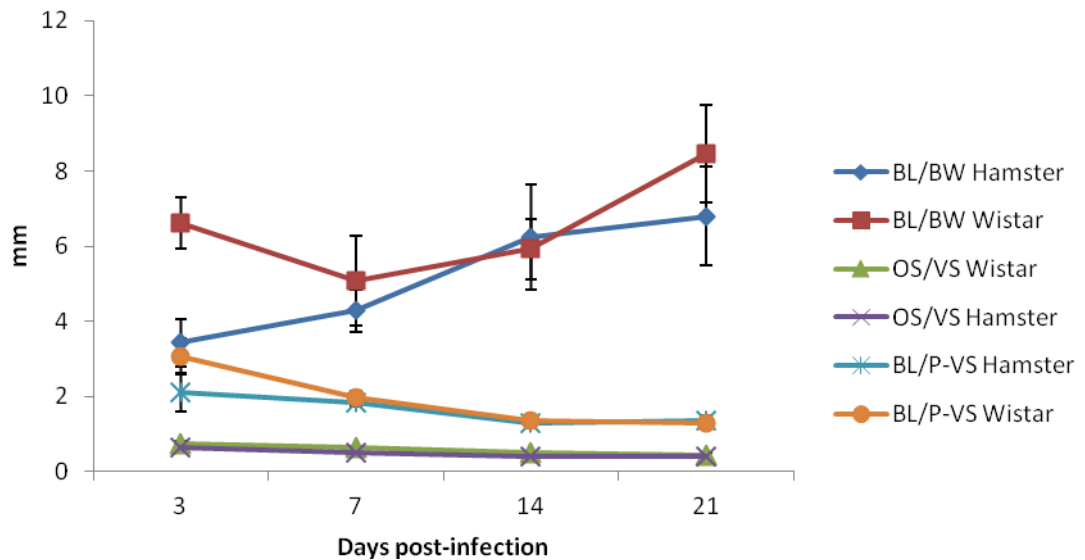
At day 3, the morphometric evaluation was not performed because gonads were not developed (Table 1). The gonads showed similar length and width in the initial phase of developmental (7days pi), after this, the mean of growth on each gonad was significant in all periods analyzed. The posterior testis presents a significant increase in the growth rate than the anterior testis in hamster. Comparatively, testes and ovary showed similar pattern of growth both in hamsters and rats. The comparison of morphometric data on the means of gonads of worms indicated greater development in hamster than in rat, (Table 1).

Table 1. Comparison of the measurements of the gonads of *Echinostoma paraensei* collected from hamster and Wistar rat according to age of infection (Mean ± SD)

Variable	3dpi		7dpi		14dpi		21dpi	
	Hamster	Rat	Hamster	Rat	Hamster	Rat	Hamster	Rat
ATL	-	-	0.044 ± 0.01	0.030 ± 0.008*	0.393 ± 0.13	0.278 ± 0.08*	0.522 ± 0.12	0.294 ± 0.06 ***
ATW	-	-	0.061 ± 0.01	0.032 ± 0.009 ***	0.368 ± 0.14	0.224 ± 0.13**	0.610 ± 0.02	0.358 ± 0.08 ***
PTL	-	-	0.046 ± 0.007	0.027 ± 0.005***	0.582 ± 0.16	0.324 ± 0.09**	0.646 ± 0.09	0.403 ± 0.09 ***
PTW	-	-	0.061 ± 0.01	0.032 ± 0.009***	0.599 ± 0.09	0.30 ± 0.11***	0.586 ± 0.08	0.376 ± 0.05 ***
OL	-	-	0.054 ± 0.01	0.035 ± 0.005***	0.262 ± 0.06	0.174 ± 0.06*	0.333 ± 0.05	0.179 ± 0.04 ***
OW	-	-	0.058 ± 0.01	0.032 ± 0.004***	0.432 ± 0.09	0.272 ± 0.17*	0.533 ± 0.11	0.322 ± 0.06 ***

ATL – anterior testis length; ATW – anterior testis width; PTL- posterior testis length; PTW- posterior testis width; OL- Ovary length; OW – ovary width; (-) unmeasured;
(*= significant; **= very significant; ***= extremely significant).

3.3. Comparison of the morphological data between hamster and rat

At three days of age, the body was oval to elongate with slender posterior region, ventral sucker located at middle of the body and some worms had posterior region post ventral sucker tapered. The genital anlage was visible, while some worms showed the column of cells linking the genital anlage to cirrus' sac primordium (data not shown).

At seven days of age, worms collected from hamster showed peristomic collar armed with spines, muscular oral and ventral sucker in the middle region of the body (Figure 5a), pre pharynx, muscular pharynx and long esophagus bifurcating anterior to the ventral sucker at the level of the cirrus sac (Figure 5a). The cirrus sac is small composed by a set of cells differentiated of the parenchyma's cells containing an initial muscular structure (Figure 5a). The male (anterior and posterior testis) and female gonads (straight uterus and ovary) were individualized structure located in the posterior region of the body (Figure 5b). Worms collected from rat showed ventral sucker in the middle region of the body (Figure 5c). A morphological integument abnormality characterized by a parenchyma extrusion toward the outer region was detected in about 30% of flukes examined from rat (Figure 5c). Images taken in the posterior end showed poorly developed gonads (Figure 5d).
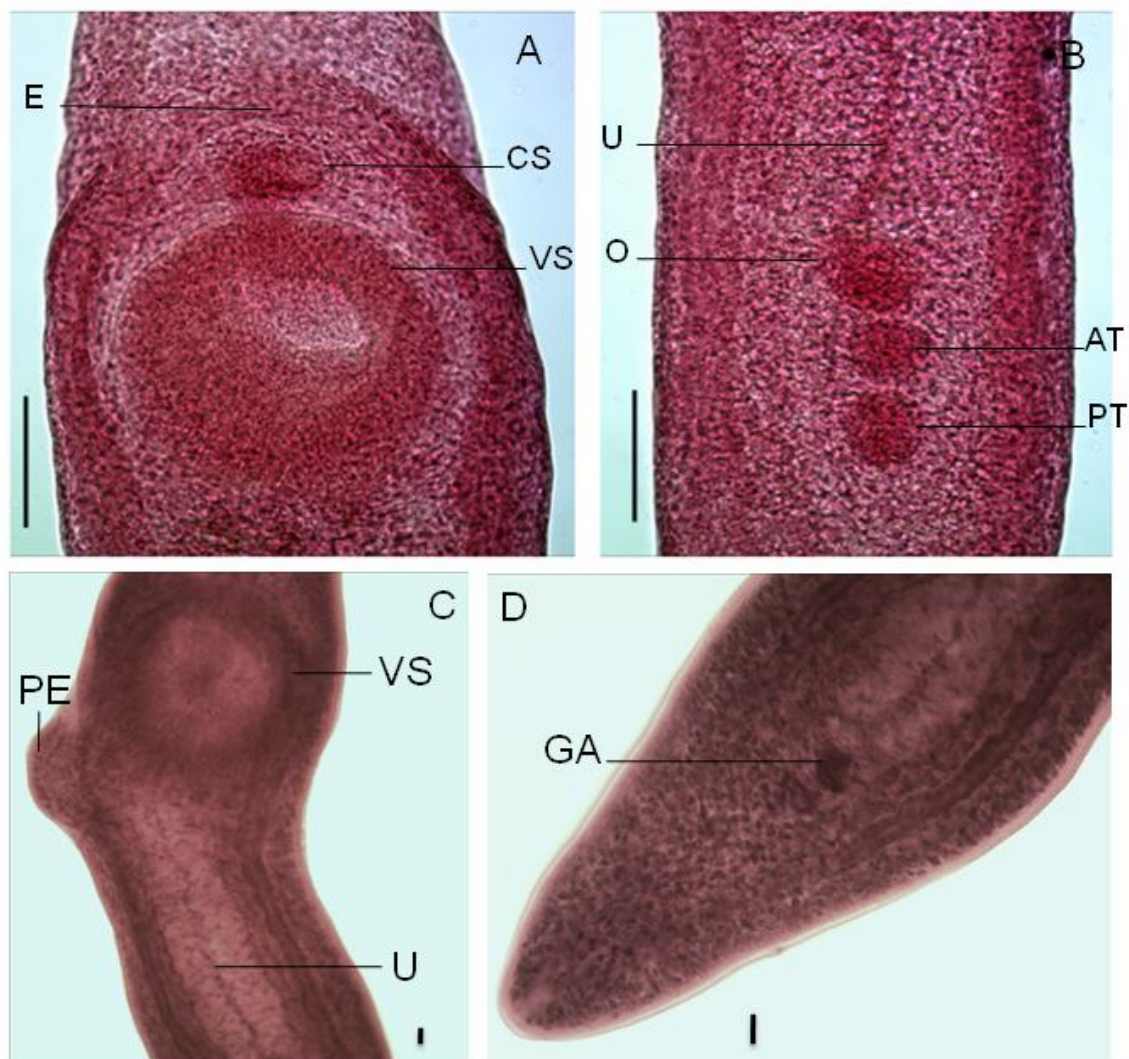
Figure 5 – Light microscopy of *Echinostoma paraensei* collected from golden hamster (A,B) and rats (C,D) 7 days post infection stained with hydrochloric carmine. (A) Middle region of the body showing esophagus [E], cirrus sac [CS] and ventral sucker [VS]; Scale bars: 25µm (B) posterior region of the body showing uterus [U], ovary [O] anterior testis [AT] and posterior testis [PT]. Scale bars: 25µm. (C) Middle region of the body showing ventral sucker [VS] and parenchyma extrusion [PE]; Scale bar: 10 µm (D) Posterior region of the body showing undeveloped gonads as genital anlage [GA]. Scale bar: 10 µm.

At fourteen days of age, most digestive, adhesion and gonads were formed. At this stage, 89% of worms derived from hamster were ovigerous. The ventral sucker was located in the first third of the body length. Cirrus' sac lying transversely anterior to larger ventral sucker containing seminal vesicle and developed cirrus were found (Figure 6a). Developing eggs were observed in the outset of the uterus located between the ovary and anterior testis (Figure 6b). The number of uterine eggs ranged 29-196 (103). Vitelline glands were laterally distributed on the body wall from the ventral sucker reaching the posterior end of the body (Figure 6c). Images taken from worms harvested from Wistar rat showed ventral sucker and cirrus sac in the anterior end (Figure 6a). Most worms (87.5%) were not ovigerous. Testes were larger than undeveloped ovary, whereas uterine eggs were not evidenced (Figure 6b). The vitelline glands were not developed (Figure 6c).

At 21 days post infection, worm recovery was low (8%) in rat. Those worms were characterized by the presence of developed organ, however 20% worms from rat were not gravid. There were few uterine eggs in ovigerous flukes, lesser than hamster.
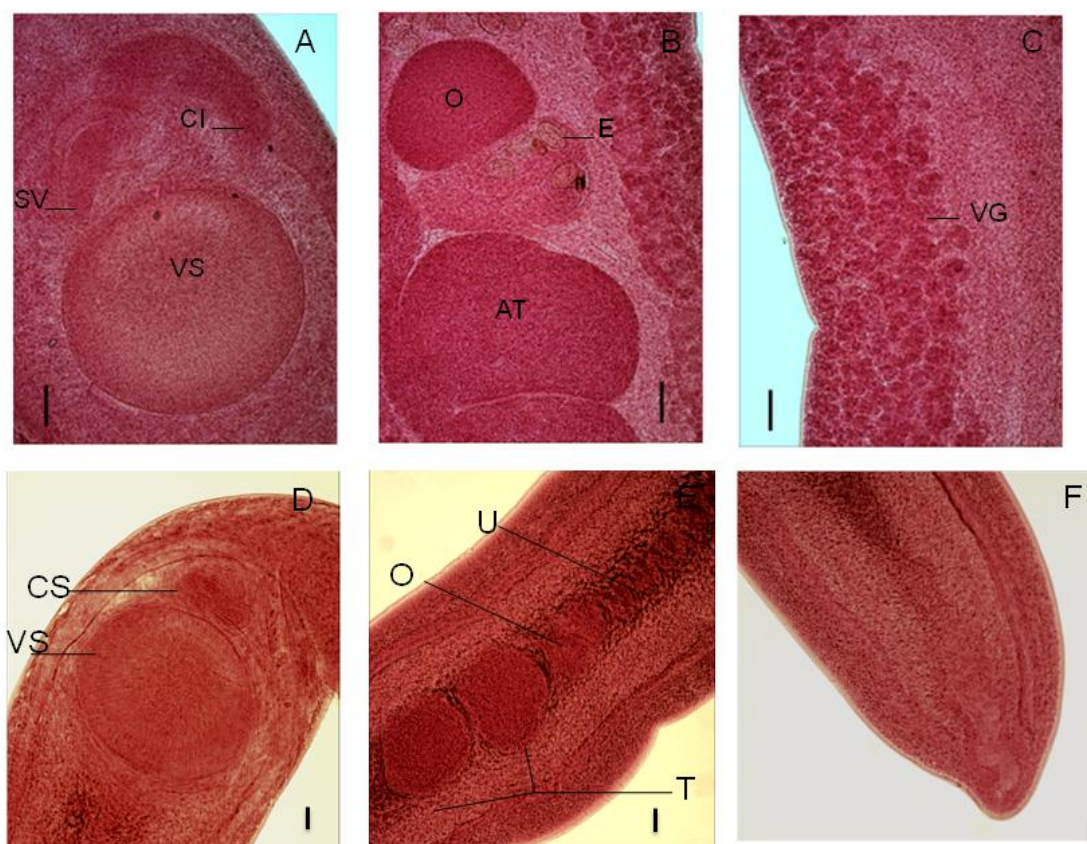
Figure 6 – Light microscopy of *Echinostoma paraensei* collected from golden hamster (A, B, C) and rats (D,E,F) 14 days post infection stained with hydrochloric carmine . (A) Ventral sucker [VS], cirrus pouch containing seminal vesicle [SV] and cirrus [CI]; Scale bars: 50µm (B) Developing eggs [E] located between ovary [O] and anterior testis [AT]; Scale bars: 50µm (C) Vitelline glands laterally distributed on the body wall. Scale bars: 50µm. (D) Anterior end showing cirrus sac [CS] and ventral sucker [VS]; Scale bar: 50 µm (E) Two testes [T], ovary and uterus [U]; Scale bar: 50 µm (F) Posterior region of the body without vitelline glands. Scale bar: 50 µm.

3.4. Confocal laser scanning microscopy examination

The phenotype of 21-days old worms collected from hamster displayed the characteristic echinostomatid collar, prominent suckers and tegument spines (Figure 7a). The spine collar is disposed in a single row at the anterior end of the body interrupted at the center of the ventral face (Figure 7a). The tegument between suckers presents spines (Figure 7a). The oral sucker was spherical sub terminal composed by meridional and radial muscles (Figure 7b). Muscular pharynx composed by circular muscles (Figure 7c). The ventral sucker is well developed spherical wide, located in the in the first third of the body with an oval aperture without papillae. The ventral sucker larger than oral sucker is composed by circular bundles muscles (Figure 7d). The uterus opens anterior to the ventral sucker in the cirrus sac with intricate network of muscles insertion (Figure 7d).

The male reproductive system consists of two testes and a cirrus sac containing prostatic gland, seminal vesicle, ejaculatory duct and a muscular cirrus. The ovoid cirrus sac is made up of several morphologically different portions (Figure 8a). The distal region of the cirrus sac is enlarged and stores matted sperm in a seminal vesicle (Figure 8b). A duct leaves from the seminal vesicle and enlarge forming a chamber located just anterior to ejaculatory duct and cirrus named bulb of ejaculatory duct (Figure 8c). This chamber is bordered inside by a syncytial cells with the morphology similar to secretor cells (Figure 8c). No glandular material was seemed in this organ. The cirrus is robust found extend or introverted. There is an ejaculatory duct in the cirrus formed by highly circular and longitudinal muscles (data not shown).

Figure 7 - Confocal laser scanning microscopy images of *Echinostoma paraensei* collected from golden hamster 21 days post infection stained with hydrochloric carmine. (A) Anterior end with peristomic collar [PC] and tegumentar spines [TS]. (B) Oral sucker with meridional muscles [mm] and radial muscles [rm]. (C) Pharynx composed by circular muscles [cm] (D) Gonopore [Gp] surrounded diagonal muscles [DM] is located below the ventral sucker [VS].

Figure 8 - Confocal laser scanning microscopy images of *Echinostoma paraensei* collected from golden hamster 21 days post infection stained with hydrochloric carmine. (A) Panoramic view of the cirrus sac showing seminal vesicle [SV], bulb of the ejaculatory duct [BED], ejaculatory duct [ED] in the cirrus and secretor cells [SC]. (B) Seminal vesicle [SV] with abundant sperm [SpP]. (C) Seminal vesicle [SV] forming a duct and a bulb of the ejaculatory duct [BED].

The female reproductive system contains an ovary and curly uterus connected to glandular structures as seminal receptacle, Mehli's gland and ducts (oviduct, ovovitellin ducts, vitellin duct and Laure's canal). The ovary is oval and smooth, transversely situated in the body and just anterior to anterior testis (Figure 9a). Ovary mostly formed by hexagonal cells with large nucleus (oocytes) which fill loosely at the center and around while in its periphery formed by few small immature cells (Figure 9a,b). The ovary has a lateral interruption on its periphery forming the ovicapt (Figure 9b). The ovicapt communicated with a enlarge region of the oviduct which allows the release of oocyte from the ovary. There are two transverse vitelline ducts fully by viteline developed cells (Figure 9c). The vitelline cells carried are stored in the vitelline reservoir (Figure 9d), so taken in oviduct by ovoviteline duct (Figure 9c). After 21 days pi, the vitelline reservoirs, vitelline ducts, and ovovitelline duct are all completely fully

with vitelline cells. Vitelline glands is situated on the lateral fields since the region next to the ventral sucker until the posterior end of body as a "cluster of grapes" formatted for columnar cells with a central nucleus of irregular size (Figure 9e). Each individualized vitelline glands is composed by cells with large central nucleus and accumulation of the granules in the periphery of the cytoplasm (Figure 9f). The oviduct is long and narrow containing bundles of in line sperm (Figure 10a). The final portion of oviduct joins to uterus which receives the eggs (Figure 10a). Vitelline glands synthesize vitelline follicles which appear like prominent cellular inclusion (Figure 10b,c). The uterus is wide and shows convolution with sperm mixed with eggs (Figure 10a,c) or is invariably filled by hundreds eggs. The uterus finishes in a genital pore located in the ventral surface of the body immediately anterior to the ventral sucker at level of the cirrus sac.
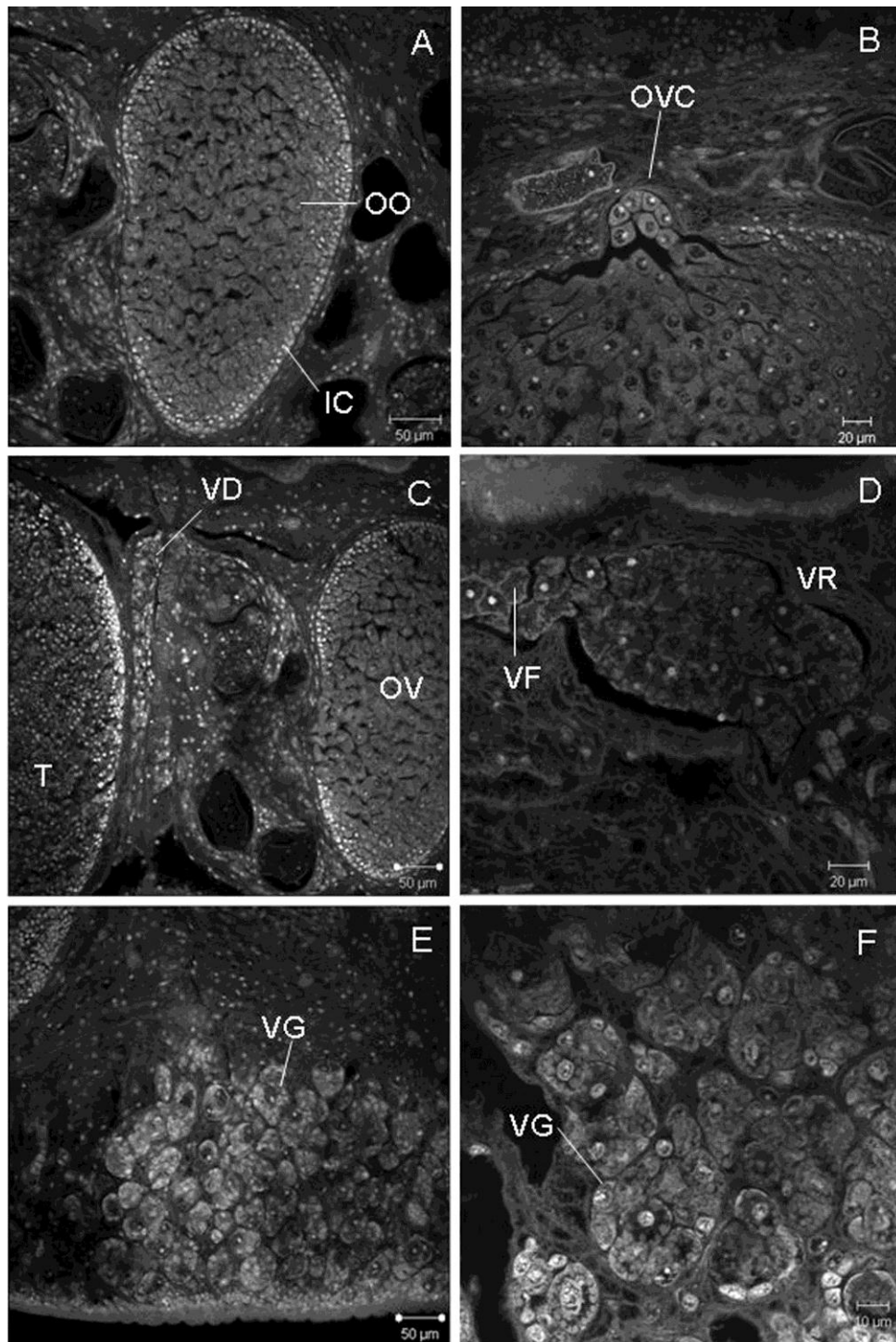
Figure 9 - Confocal laser scanning microscopy images of female gonads of *Echinostoma paraensei* collected from golden hamster 21 days post infection stained with hydrochloric carmine. (A) Ovary composed by oocytes [OO] and immature cells [IC] in the periphery. (B) Periphery of the ovary showing the ovicapt [OVC] with oocytes. (C) Vitelline ducts [VD] containing vitelline follicles [VF] located between ovary [OV] and testis [T]. (D) Vitelline reservoir [VR] storing vitelline follicles [VF]. (E) Vitelline glands [VG]. (F) Vitelline glands composed by a large nucleus and prominent inclusion of vitelline follicle.
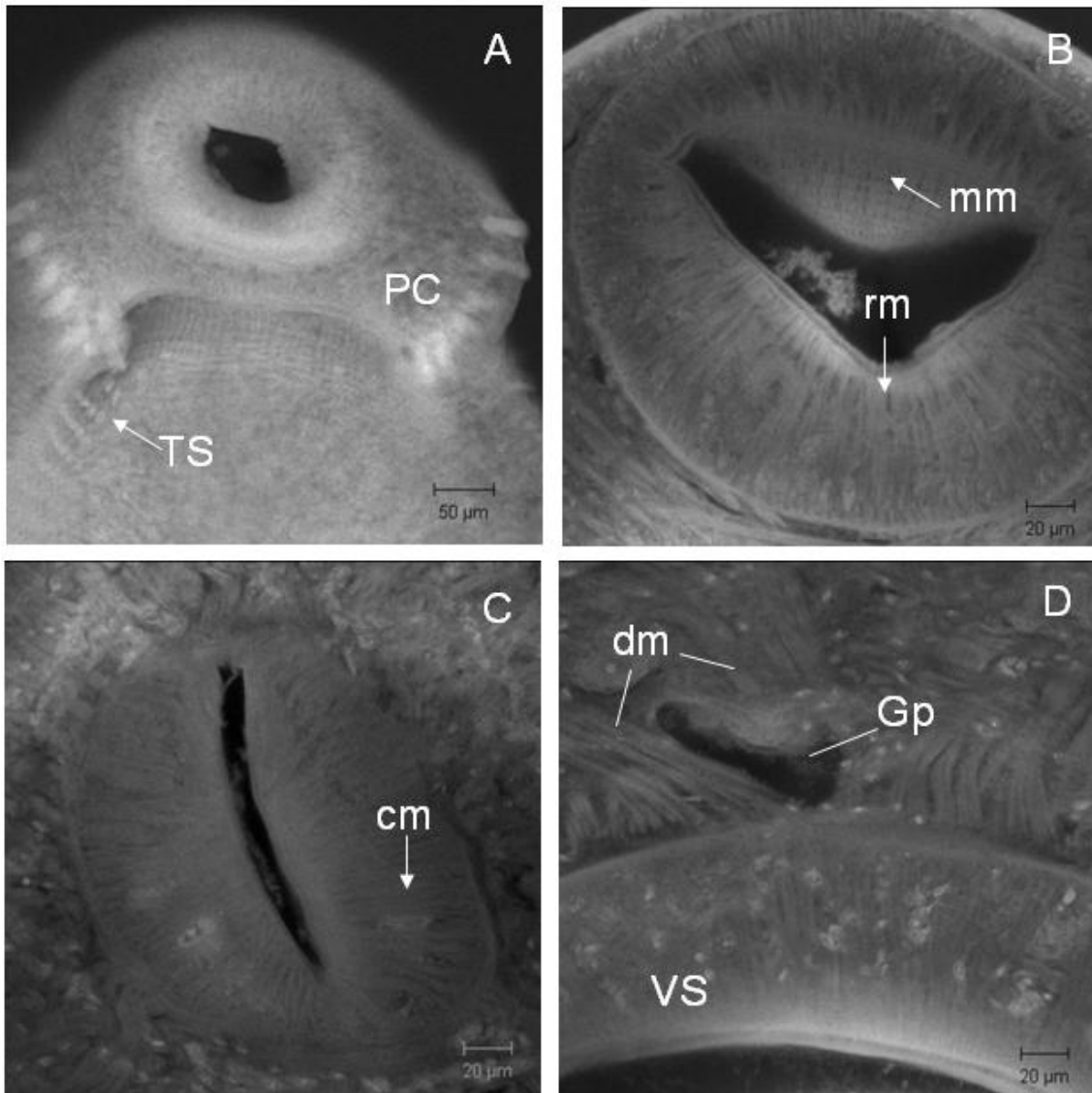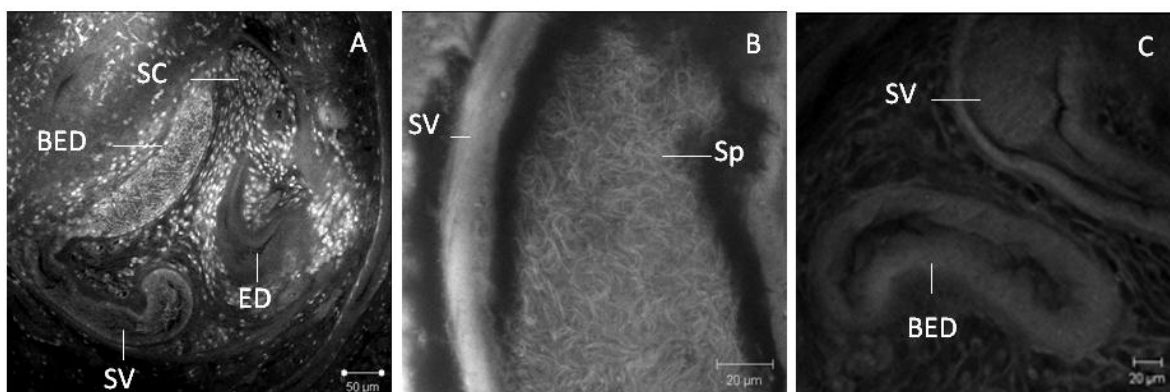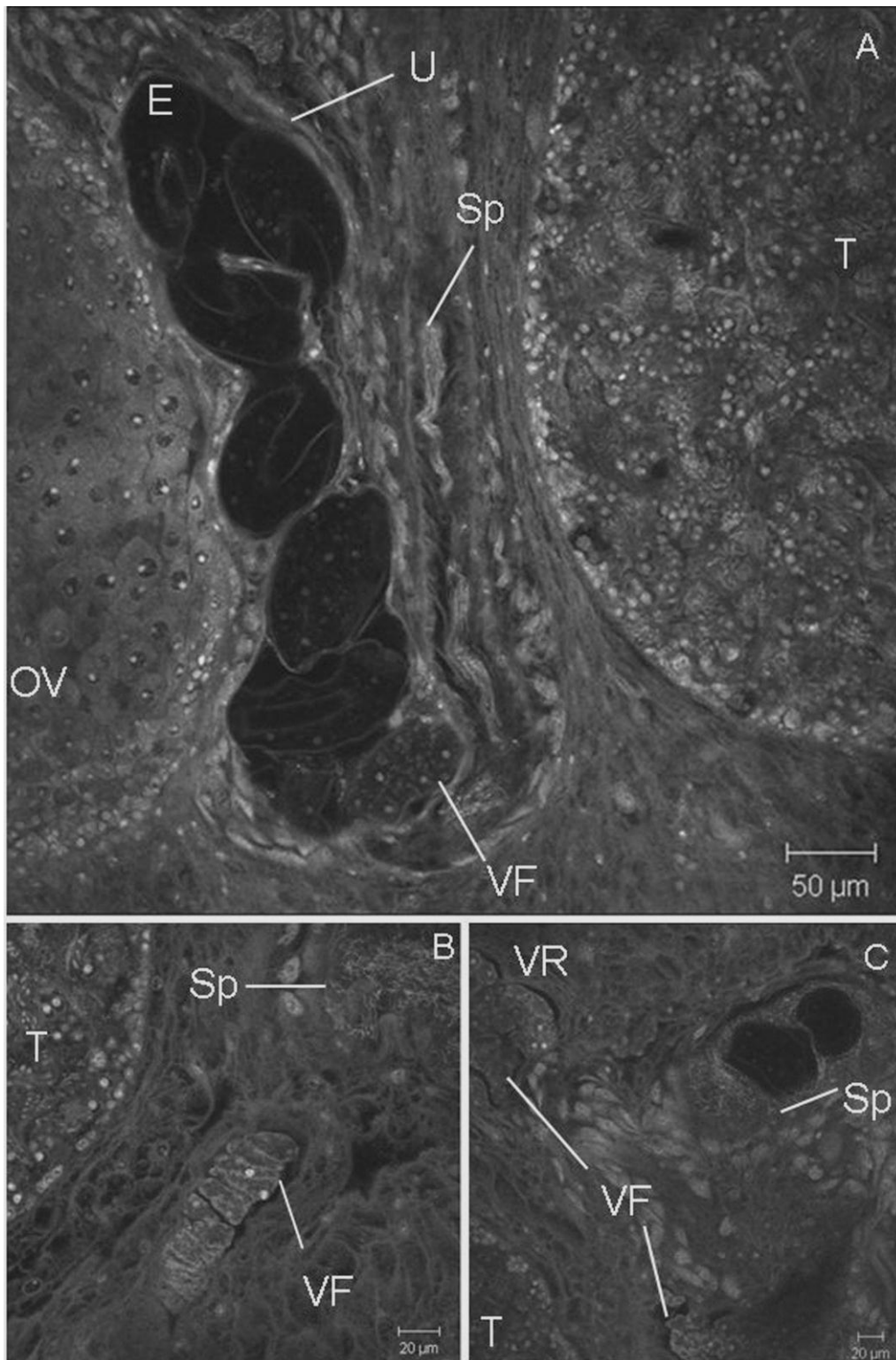
Figure 10 - Confocal laser scanning microscopy images of gonads of *Echinostoma paraensei* collected from golden hamster 21 days post infection stained wit hydrochloric carmine. (A) Oviduct with bundles of sperm [Sp], ovary [OV], testis [T] and uterus [U]

with developing eggs [E]. (B) and (C) Vitelline reservoir [VR] with vitelline follicles [VF] and sperm [Sp] in the proximal region of the uterus containing developing eggs.

Concerning worms collected from rat 21 days post infection, confocal examination revealed absence of vitelline glands and low developed rate of oocytes in the ovary (Figure 11a,b). However, the testis showed a normal spermatogenesis with sperm stored in the seminal vesicle (Figure 11c).
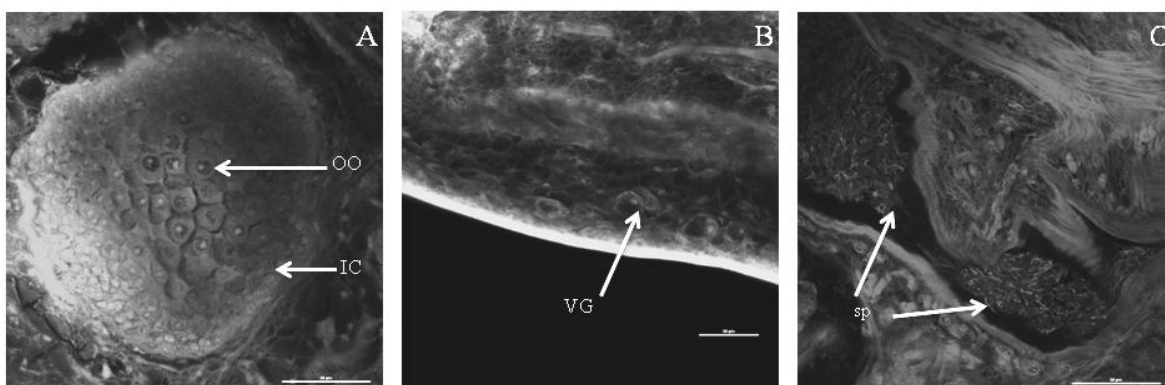
Figure 11 - Confocal laser scanning microscopy images of gonads of *Echinostoma paraensei* collected from Wistar rat 21 days post infection stained with hidrochlorich carmine (A) Ovary with developed oocytes [OO] and immature cells [IC]. (B) Undeveloped vitelline glands [VG]. (C) Sperm [SP] stored in the seminal vesicle. Scale bar: 50µm.

4. Discussion

Investigation of growth and development of *Echinostoma* species (Mohandas and Nadakal., 1978; Seo et al., 1985; Franco et al., 1986; Fried et al., 1988; Toledo et al., 2004a,b) in vertebrate hosts is of importance, and *E. paraensei* appears to be well suited for such studies. The probability of parasite establishment within the host and its longevity can vary considerably between individual hosts. Thus, high susceptible hosts carry the infection for a long-term in contrast to other less susceptible, in which the

outcome of infection is less successful (Mahler et al., 1995; Toledo et al., 2004a,b; Mahler et al., 2005; Toledo et al., 2006). It is known that permissive hosts like golden hamster and mice provide a favorable environment for parasites, whilst Wistar rats do not, allowing their classification as less compatible host (Muñoz-Antoli et al., 2014). Experimental evidences showed that *E. paraensei*-infected Wistar rats decrease worm burden by week 2 after infection and two weeks later, develop self-cure (Garcia et al., 2011).

Three main questions arise from this issue. First, at what time *E. paraensei* begins the process of adaptation in the host? Upon infecting a warm-blooded mammalian host, *E. paraensei* metacercariae undergoes physiological and morphological transitions, indicating adaptive mechanisms for living in the new biological micro-environment. In other words, invading parasites have the ability to perceive a diversity of signals from environmental parameters. However, this issue has not been explored among hosts differing in their host-parasite relationship. Could the degree of permissiveness influence the development of juvenile stages? To answer this question, the early step to know was the phenotypic features of infecting stages. We recently overlap this challenge using *E. paraensei* newly excysted metacercariae (Souza et al., 2013).

Second, to test the hypothesis that different degree of susceptibility could be impair the morphogenesis of the reproductive system in older parasites, we extended our previous studies (Souza et al., 2011), comparing the phenotypic characteristics of worms recovered from hamster and rat from 3 to 21 days post infection. Based on this approach, we also investigated if the variation in susceptibility could impair the allometric phenotypic traits. In an attempt to clarify this issue, the Body length/ body

width, oral sucker width/ventral sucker width, body length/posterior end of body to ventral sucker ratios were carried out. The earliest ontogenic stages (3 and 7-day old worms) preserved a similar pattern of development. To the best of our knowledge, the present allometric study is the first assessment providing morphological traits in juvenile parasite recovered from compatible and less compatible hosts. Unlike other studies based on *F. hepatica* adult worm allometry, our findings did not detect available phenotypic ratios for distinguishing different vertebrate hosts (Valero et al., 2001). It is worth to note that laboratory studies indicated that adult worms show positive allometric growth in *E. miyagawai* and negative allometry in *E. revolutum* (Kostadinova et al., 2000).

Third, the determination of major event involved in reproductive organ differentiation is fundamental for understanding the reproductive biology of *E. paraensei* in vertebrate hosts (Nollen, 1997). Even though 3-day old worms showed similar growth, the recovery rate was slightly higher in hamster (15%) than rat (12%). From day 7 onwards, worms grew almost 27-fold compared to the initial size. In fact, published studies have evidenced an initial lag phase (first 3 dpi) in the development followed by a high growth rate similar to this study (Mohandas and Nadakal, 1978; Yao et al., 1991; Manger and Fried, 1993; Ursone and Fried, 1995). It should be highlighted however, that worms derived from rat had smaller body size, integument changes and poorly developed gonads compared to hamster. Consistent with this, we previous demonstrated that *Schistosoma mansoni* female worms recovered from rats also failed to develop the reproductive system (Silva-Leitão et al., 2009). Concerning the worm recovery, it increased compared with 3-day old worms, however a more pronounced difference between hamster (37%) and rat (8%) was found.

It remains to be determined how low permissiveness influences the reproductive system of *Echinostoma* immature worms. In this investigation, worms harvested from hamster showed normal gonads and vitelline glands laterally distributed on the body. This finding is in line with previous studies, indicating that the reproductive system is functional on 14 dpi in hamsters (Souza et al., 2011). In contrast, most worms from rat were not ovigerous, uterine eggs were not evidenced and vitelline glands were not developed. Based on the fact that the host-parasite relationship is influenced by a bidirectional communication (Kusel et al., 2007), our plausible explanation is that rat may be impaired to modulate growth and organ maturation in response to host signal.

Strikingly, host-induced morphological alterations have been described in echinostome derived from hostile environment. For instance, *E. trivolvis* adult worms show retraction or loss of collar spines (Kruse et al., 1992), whilst *E. caproni* also shows retraction of collar spines (Fujino et al., 1994). Moreover, *E. caproni* adult worms collected from rats (low compatible host) showed an increasing in the density of spines in the oral sucker area compared to mice (compatible host). Differences regard the tegumentary spines were also evidenced. Rats showed spines sharper, whereas those from mice were larger and blunter (Sotillo et al., 2010).

Furthermore, by 14 days post infection, worm recovery in rat decreased (20.6%) compared to hamster (36%). This study and others (Garcia et al., 2011) clearly demonstrate that rat is non-permissive host environment because *E. paraensei* has limited survival. In contrast, it appears that rat albino is permissive to *E. hortense* because the life span ranges 6 up to 49 days pi (Seo et al., 1985). Laboratory studies have pointed out that various immune effectors and intestinal tissue disorder may be

implicated in worm release from the gut (Weinstein & Fried, 1991; Toledo et al., 2006; Trelis et al., 2011; Cortés et al., 2015).

After 21 days of infection, worm recovery declined to 8% in rat, whilst remained stable in hamster (36%). The data gathered in this phase provides complementary insight into the phenotypes of worms expressed under favorable and hostile microenvironment. Worms collected from hamster presented near 2-fold size than rats, ratios measurements and all gonads measurements were greater than those from rats. Focusing on the male gonads, we noted normal spermatogenesis with sperm stored in the seminal vesicle and precocious maturity than female. This reproductive strategy may be implicated to ensure successful reproduction among hermaphrodite flukes. Interestingly, previous studies also revealed this reproductive behavior in *S. mansoni*, which has different gonochoric sexual system (separate sexes) (Silva-Leitão et al., 2009). In contrast, worms from rats were not able to reach sexual maturation with signs of atrophy of the gonads represented by absence or underdeveloped vitelline glands, low developed rate of oocytes in the ovary and uterine egg concentration fewer than hamster.

Most studies on the reproductive biology among flatworms were conducted in the family Schistosomatidade, whose adult worms are gonochoristics (Despres and Maurice, 1995). Interestingly, the proliferation and differentiation of cells in the reproductive organs ovary and vitellelline depend on the constant pairing contact, in which the male activates signal transduction pathways in the female (Kunz, 2001). In previous studies, our group showed that immature females collected from permissive host (mice) possess small oocytes representing an immature stage without differentiation (oogonia) (Neves et al., 2005). Considering that the seminal vesicle was

not empty, it could be possible to transfer its contents to the female reproductive system. Unfortunately, no image performed by confocal microscopy evidenced sperm in the receptaculum seminal. Thus, future experiments are needed to clarify this intriguing issue. In conclusion, to the best of our knowledge, this is the first appointment demonstrating that low permissiveness influences the reproductive system of echinostome since the immature stages of development. The phenotypic analysis evidenced that hamster provides a more favorable microenvironment for gonads development than rat, confirming golden hamster as a permissive host, whereas Wistar rat is less permissive host.

References

Bonfim TCS, Maldonado A, Tunholi VM, Tunhol-Alves VM, Faro MJ, Mota EM, Barros TCS, Pinheiro S, Garcia SJ. 2014. Biochemical and histhological alterations in Biomphalaria glabrata due to co-infection *Angiostrongylus cantonensis* and *Echinostoma paraensei*. J Invertebr Pathol. 115, 80-85.

Ellery, AW. 1985. Guidelines for specification of animals and husbandry methods when reporting the results of animal experiments. Working Committee for the Biological Characterization of Laboratory Animals/GV-SOLAS. Laboratory Animals 19, 106–108.

Faltýnková A, Georgieva S, Soldánová M, Kostadinova A. 2015.A re-assessment of species diversity within the 'revolutum' group of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) in Europe. Syst Parasitol. 90(1):1-25

Ferraz J, Souza J, Costa-Silva M, Torres E, Santana A, Lanfredi R, Maldonado A, Garcia J. 2012. Effect of praziquantel on adult *Echinostoma paraensei* worms in experimentally infected mice. Parasitol Res 111:143-148.

Franco J, Huffman JE, Fried B. 1986. Infectivity, growth and development of *Echinostoma revolutum* (Digenea: Echinostomatidae) in the golden hamster, *Mesocricetus auratus*. J. Parasitol 72, 142-147.

Fried B, Graczyk TK. 2004. Recent advances in the biology of *Echinostoma* species in the "revolutum" group. Adv Parasitol 29, 163-210.

Fried B, Reddy A (1997). Comparative studies on excystation and morphological features of the metacercariae of *Echinostoma paraensei* and *E. caproni*. Int J Parasitol 27:889-901.

Fried B, Donovick RA, Emili S. 1988. Infectivity, growth and development of *Echinostoma lie* (Trematoda) in the chick. Parasitology 82:413-414.

Fujino T, Nakano T, Washioka H, Tonosaki A, Ichikawa H, Fried B. 2000. Comparative ultrastructure of eggs in *Echinostoma paraensei*, *E. caproni*, and E. *trivolvis* (Trematoda: Echinostomatidae). Parasitol Res 86:427-30.

Garcia JS, Maldonado A, Bidau JC, Correa LR, Lanfredi RM, Coelho PMZ. 2010. Effect of early infection with *Echinostoma paraensei* on interaction *Schistosoma mansoni* with Biomphalaria glabrata and Biomphalaria tenagophila. Mem Inst Oswaldo Cruz. 105, 499-503.

Garcia JS, Hooper CS, Simões RO, Santos MAJ, Maldonado A, Silva JP. 2011. Biochemical and histological responses of *Rattus norvegicus* (Wistar) infected with *Echinostoma paraensei* (Trematoda: Echinostomatidae). Vet Parasitol. 178:86-92.

Garcia JS, Pinheiro S, Hooper CS, Simões RO, Ferraz JS, Maldonado A. 2012. Haematological alterations in *Rattus norvegicus* (Wistar) experimentally infected with *Echinostoma paraensei* (Trematoda: Echinostomatidae). Exp Parasitol 131:300-303.

Georgieva S, Faltýnková A, Brown R, Blasco-Costa I, Soldánová M, Sitko J, Scholz T, Kostadinova A. 2014. *Echinostoma revolutum* (Digenea: Echinostomatidae) species complex revisited: species delimitation based on novel molecular and morphological data gathered in Europe. Parasit Vectors. 27;7(1):520.

Gibson DI, Bray RA, Hunt D, Georgiev BB, Scholz T, Harris PD, Bakke TA, Pojmanska T, Niewiadomska K, Kostadinova A, Tkach V, Bain O, Durette-Desset MC, Gibbons L, Moravec F, Petter A, Dimitrova ZM, Buchmann K, Valtonen ET, de Jong Y. 2014. Fauna europaea: helminths (animal parasitic). Biodivers Data J. 17:e1060

Gonçalves JP, Oliveira-Menezes A, Maldonado Junior A, Carvalho TM, de Souza W. 2013. Evaluation of Praziquantel effects on *Echinostoma paraensei* ultrastructure.Vet Parasitol 194: 16-25.

Hansen K, Nielsen JW, Hindsbo O, Christensen NO. 1991. *Echinostoma caproni* in rats: worm population dynamics and host blood eosinophilia during primary 6, 25 and 50 metacercarial infections, and resistance to secondary and superimposed infections. Parasitol Res. 77: 686-690.

Leles D, Cascardo P, Friere Ados S, Maldonado A, Sianto L, Araújo A. 2014. Insight about echinostomiasis by paleomolecular diagnosis. Parasitol Inter 63, 646-649.

Lie KJ and Basch PF. 1967. The life history of *Echinostoma paraensei* sp. n. (Trematoda: Echinostomatidae). J Parasitol 53, 1192-1199.

Machado-Silva JR, Pelajo-Machado M, Lenzi HL, Gomes DL. 1998. Morphological study of adult male worms of *Schistosoma mansoni* by confocal laser scanning microscopy. Mem Inst Oswaldo Cruz. 93, 303-307.

Maldonado A, Loker ES, Morgan JAT, Lanfredi RM. 2001a. Description of the adult worms of a new Brazilian isolate of *Echinostoma paraensei* (Platyhelminthes: Digenea) from its natural vertebrate host *Nectomys squamipes* by light and scanning electron microscopy and molecular analysis. Parasitol Res. 87: 840-848.

Maldonado A, Vieira GO, Garcia JS, Rey L, Lanfredi RM. 2001b. Biological aspects of a new of isolate of *Echinostoma paraensei* (Trematoda: Echinostomatidae): susceptibility of sympatric snails and the natural vertebrate host. Parasitol Res 87: 853-859.

Maldonado Jr., A., Vieira, G.O., Lanfredi, R.M., 2003. *Echinostoma luisreyi* n. sp. (Platyhelminthes: Digenea) by light and scanning electron microscopy. J Parasitol 89:800–808.

Maldonado Jr., A., Zeitone, B.K., Amado, L.A., Rosa, I.F., Machado-Silva, J.R., Lanfredi, R.M., 2005. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. J Helmintol 79, 345–351.

Manger PM, Fried B. 1993. Infectivity, growth and distribution of preovigerous adults of *Echinostoma caproni* in ICR mice. J. Helminthol. 67, 158-160.

Mohandas A, Nadakal AM. 1978. In vivo development of *Echinostoma malayanum* Leiper, 1911 with notes on effects of population density, chemical composition and pathogenicity and *in vitro* excystment of the metacercariae (Trematoda: Echinostomatidae) Z. Parasitenkd. 55, 139-151.

Muñoz-Antoli C, Cortés A, Torres D, Esteban JG, Toledo R. 2014. Haematological changes in the laboratory rat *Rattus norvegicus* infected with *Echinostoma caproni* (Trematoda: Echinostomatidae). J Helminthol 3,1-5.

Pinheiro P, Maldonado A. Attias M, Landrefi RM. 2005. Ultraestruture of the miracidium of *Echinostoma paraensei* Lie and Basch, 1967 (Trematoda: Echinostomatidae). Parasitol Res 97, 367-372.

Seo, BS Chun, KS, Chai, JY, Hong, SJ, Lee, SH, 1985. Studies on intestinal trematodes in Korea XVII. Development and egg laying capacity of *Echinostoma hortense* in albino rats and human experimental infection. J Parasitology 23, 24–32.

Souza JGR, Garcia JS, Manso PPA, Neves RH, Maldonado A, Machado-Silva JR. 2011. Development of the reproductive system of the *Echinostoma paraensei* in *Mesocricetus auratus* analyzed by light and confocal scanning laser microscopy. Exp Parasitol 128:341-346.

Silva-Leitão FW, Biolchini CL, Neves RH, Machado-Silva JR. 2009. Development of *Schistosoma mansoni* in the laboratory rat analyzed by light and confocal laser scanning microscopy. Exp Parasitol 123, 292-295.

Souza JGR, Garcia JS, Neves RH, Machado-Silva JR, Maldonado A. 2013. *In vitro* excystation of *Echinostoma paraensei* (Digenea: Echinostomatidae) assed by light microscopy, morphometry and confocal microscopy. Exp Parasitol 4:701-707.

Toledo R, Espert A, Carpena I. 2004a. The comparative development of *Echinostoma caproni* (Trematoda: Echinostomatidae) adults in experimentally infected hamsters and rats. Parasitol. Res 93, 439-444.

Toledo R, Espert A, Muñoz-Antoli C, Marcilla A, Fried B, Esteban JC. 2004b. Kinetic of *Echinostoma caproni* (Trematoda: Echinostomatidae) antigens in feces and serum of experimentally infected hamster and rats. J. Parasitol 90, 752-758.

Toledo R, Fried B. 2005. Echinostomes as experimental models for interactions between adult parasites and vertebrate host. Trends Parasitol 21:251-254.

ToledoR, Monteagudo C, Espert A, Fried B, Esteban JG, Marcilla A. 2006. *Echinostoma caproni*: Intestinal pathology in the golden hamster, a highly compatible host, and the wistar rat, a less compatible host. Exp Parasitol 112:164-171.

Ursone RL, Fried B. 1995. Light and scanning electron microscopy of *Echinostoma caproni* (Trematoda) during maturation in ICR. Parasitol Res 81:45-51.

Valero MA, Darce NA, Panova M, Mas-Coma S. 2001. Relationship between host species and morphometric pattern in *Fasciola hepatica* adults and eggs the northern Bolivian Altiplan hyperendemic region. Vet Parasitol 102:85-100.

Yao G, Huffman JE, Fried B. 1991. The effects of crowding on adults of *Echinostoma caproni* in experimentally infected golden hamsters. J Helmintol 65, 248–254.

3 *IN VITRO* EXCYSTATION OF *ECHINOSTOMA PARAENSEI* (DIGENEA: ECHINOSTOMATIDAE) METACERCARIAE ASSESSED BY LIGHT MICROSCOPY, MORPHOMETRY AND CONFOCAL LASER SCANNING MICROSCOPY

Joyce Souza[a], Juberlan Garcia[b], Renata H Neves[a], José Roberto Machado-Silva[a/+], Arnaldo Maldonado[b]

[a] Romero Lascasas Porto Laboratory of Helminthology, Department of Microbiology, Immunology and Parasitology, School of Medical Science, Biomedical Center, Av. Prof. Manoel de Abreu 444/5º andar, Vila Isabel, 20511-070, Rio de Janeiro, Brazil.

[b] Laboratory of Biology and Parasitology of Wild Mammal Reservoirs, Oswaldo Cruz Institute, Av. Brasil 4365, Manguinhos, 21040-360, Rio de Janeiro, Brazil

[+] Corresponding author: José Roberto Machado-Silva. Phone +55 21 25876148. E-mail: machado@uerj.br

Abstract

Trypsin and bile salts have been identified as important triggers for excystation of *Echinostoma* metacercariae. Although excystation in trematodes is a well-known phenomenon, some morphological developmental changes remain to be elucidated. In order to gain further insight into the *in vitro* development of metacercariae, we assayed different cultivating conditions: 0.5% trypsin and 0.5% bile salts; 1% trypsin and 1% bile salts; 1% trypsin and 0.5% bile salts; 0.5% bile salts; or 0.5% trypsin. By means of light microscopy and confocal microscopy, we characterized each encysted, activated, breached and excysted stage based on the morphological features. However, breached

and excysted stages were not revealed in both bile salts and trypsin-free medium. Excretory concretions (25±3.9) were visualized within excretory tubules, close to the ventral sucker and genital anlage. The oral sucker armed with spines and digestive system were similar to those of adult worms. The reproductive system is composed of a genital anlage and the cirrus sac primordium. In short, trypsin and bile salts associated were fundamental for the *in vitro* metacercariae excystation of *E. paraensei*. This article presents the first detailed information of all stages of metacercariae excystation obtained through light and confocal microscopy.

Keywords: *Echinostoma paraensei*, metacercariae, excystation, *in vitro*, morphology, confocal laser scanning microscopy.

Sponsorships: CAPES.

1. Introduction

Echinostoma paraensei (Lie & Basch 1967) (Echinostomatidae: Platyhelminthes) was first described from natural infection of Brazilian *Biomphalaria glabrata* snails that were shipped to the United States (Maldonado et al. 2001). *E. paraensei* is a hermaphroditic digenetic flatworm which in the adult stage parasitizes the small intestine and bile ducts of mammals (Maldonado & Lanfredi 2009).

The semi-aquatic rodent *Nectomys squamipes* (Rodentia: Sigmondontinae) was reported to be the definitive natural host of *E. paraensei* in secluded Atlantic Forest regions of Rio de Janeiro state in Brazil (Maldonado et al. 2001). Immature eggs released from feces require appropriate environmental conditions for advancing to the next stage, giving rise to a ciliated stage, composed of miracidia that actively penetrate the first intermediate snail host, *Biomphalaria glabrata* Say (1818) (Maldonado &

Lanfredi 2009). Within this freshwater snail, the miracidia undergo asexual reproduction and produce infective cercariae. The cercariae released come into contact with a second intermediate host (*B. glabrata*), in which they penetrate and develop to the metacercarial stage in the snail's pericardic cavity (Lie & Basch 1967). Definitive hosts become infected by eating infected intermediate hosts harboring metacercariae, which excyst in the gastrointestinal tract and migrate to the small intestine, where they attach to the duodenal mucosa by the ventral sucker (Fried & Huffman 1996).

During the last three decade, advances in echinostome cultivation have evidenced that these trematodes undergo striking morphological and physiological changes (Fried & Emili 1988; Smoluk & Fried 1994; Ursone & Fried 1995; Fried & Reddy 1997; Fried et al. 2002; Fried et al. 2004; Fried & Peoples 2007; Saxton et al. 2008). In addition, bile salts and trypsin have been identified as important triggers to achieve successful excystation of *Echinostoma* metacercariae (Irwin et al. 1984; Fried & Roth 1974). *In vitro* assays have demonstrated that *E. caproni* has a higher rate of excystation than *E. trivolvis* (Fried & Emili 1988; Saxton et al. 2008) and *E. paraensei* (Fried & Reddy 1997).

Comparative morphological studies of *in vitro* excystation have demonstrated a higher number of calcareous corpuscles in *E. caproni* than in *E. paraensei* (Fried & Reddy 1997). However, the role of calcareous corpuscles (excretory concretions) is not well understood (Smoluk & Fried 1994).

Morphometric analysis has also yielded consistent results, demonstrating that the diameter of the ventral sucker and number and diameter of the excretory concretions in *E. caproni* are significantly larger than those of *E. trivolvis* (Saxton et al. 2008).

Given that there is still surprisingly little known about the excystation processes in *E. paraensei*, we decided to test whether different concentrations of trypsin and bile salts favor excystation. We further used light and confocal laser scanning microscopy images to underscore our results. We have recently reported that confocal is a useful tool to study the morphological characteristics of the reproductive system of 3-day-old worms (Souza et al. 2011). In this study, we noted four stages (encysted, activated, breached and excysted) of *E. paraensei* metacercariae. We also tested whether different concentrations of trypsin and bile salts favor staging at several time intervals. In addition, we studied the morphological characteristics of *E. paraensei* metacercariae excystation.

2 - Material and methods

2.1 - Parasites

Metacercarial cysts were obtained by dissection of the pericardium region of *B. glabrata* snails experimentally infected with *E. paraensei* (Sumidouro isolate) under a stereomicroscope. Metacercariae were grape-shaped organisms covered with a very thick translucent outer layer and a refractile inner layer. The cysts were washed in Locke's solution (9g/l NaCl, 0.4g/l KCl, 0.2g/l CacCl$_2$, 0.2g/l NaHCO$_3$) at room temperature and immediately used.

2.2 – Culture medium

The alkaline trypsin bile salts medium was prepared with 0.5% trypsin (1:250, pig pancreas, GIBCO) plus 0.5% bile salts (Sigma-Aldrich) with Earle's balanced salt solution (Earle's BSS) (6.8g/l NaCl, 0.4g/l KCl, 0.2g/l CaCl$_2$, 0.1g/l MgSO$_4$, 0.125g/l NaH$_2$PO$_4$.H$_2$O, 2.2g/l NaHCO$_3$ and 1g/l D-glucose), according to Fried and Roth (1974). Different concentrations of trypsin and bile salts were dissolved in Earle's BSS

and tested as follows: (A) 0.5% trypsin plus 0.5% bile salts; (B) 1% trypsin plus 1% bile salts; (C) 1% trypsin plus 0.5% bile salts; (D) 0.5% bile salts; and (E) 0.5% trypsin. The pH was adjusted to 8.0±0.2 with 7.5% $NaHCO_3$.

2.3 – Protocol of excystment induced *in vitro*.

Twenty metacercarial cysts were used for each tested solution in 3.5-cm Petri dishes, in a total volume of 3 mL of fresh tryspsin bile salt medium at 40 ºC and 5% CO_2 atmosphere. The determination of metacercarial staging was assessed on the basis of the morphological profile of metacercariae for each medium tested (encysted stage, activated stage, breached stage and excysted stage), as revealed by light microscopy (Smoluk and Fried, 1994). For the determination of percentage of excystation, 20 cysts were examined from each culture medium, at various intervals (15, 30, 45, 60, 90 and 120 min). Each trial was repeated three times for each medium tested. Viability of immature worms was studied using 0.5% trypan blue staining at each time interval.

2.4 - Morphology and morphometric analysis

Living metacercariae were mounted with Locke's solution on slides covered with cover slips, and then observed under a stereoscopic microscope (STEMI SV6) and drawn using a coupled Lucida camera (Zeiss Axioskop) and then photographed using a Sony DSC–S75 camera. To investigate the number of excretory concrections (calcareous concrections), 25 metacercariae maintained for 120 min in each medium were studied by light microscopy.

The excysted metacercariae used for morphological study were stained with hydrochloric carmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted as whole-mounts in Canada balsam (Neves et al. 1998). Measurements (body length and width, oral and ventral sucker diameters, spine collar

length and width, distance between anterior end of body and ventral sucker, distance between oral and ventral suckers and distance between ventral sucker and posterior extremity) were made using a Zeiss Axioskop microscope with a 40x objective. All measures are presented in micrometers. Three ratios were also calculated: (i) BL/BW, the length of the body in relation to the body width, (ii) VSL/OSL, the ventral sucker length in relation to oral sucker length, and (iii) BL/DVS, the body length in relation to the distance of the ventral sucker to posterior extremity. Measurements are presented as millimeters.

Samples were also imaged were under a Zeiss LSM 510-ZETA confocal microscope (Carl Zeiss, Germany), using a 543 nm He/Ne laser and an LP 570 filter under reflectance mode (Souza et al. 2011).

2.5 - Statistical analysis

One-way analysis of variance (ANOVA) and the post-hoc Tukey test were used to compare the mean differences of the excystment rate for each medium tested, with significance of $P<0.05$.

3 - Results

3.1 - Determination of metacercarial staging and excystation rate:

Table 1 summarizes the staging of metacercariae according to the experimental conditions. The data showed that the development kinetic depends on the concentration of trypsin and bile salts and is time-related. The metacercariae could be scored in four morphologically distinct stages of development: encysted, activated, breached and excysted.

Although encysted metacercariae were recovered from all the media tested, only that which contained 0.5% trypsin in Earle's balanced salt solution (E) reached high rate

(> 90%) of encysted metacercariae at any time of evaluation. The percentage of encysted metacercariae declined for 15-120 min in most cultures tested and concomitantly increased for the activated stage. The highest rate was obtained at 30 min, with 54, 55 and 61% of activated metacercariae in groups A, C and B, respectively.

At 45 min, we observed a high rate of breached metacercariae regardless of culture medium. Encysted and activated metacercariae cultivated in medium D and medium E were visualized. However, breached and excysted stages were not found in both bile salts and trypsin-free medium (Table 1).

Table1. Effects of different concentrations of trypsin and bile salts on staging (encysted, activated, breached and excysted) of *Echinostoma paraensei* metacercariae at different intervals.

Time (min)	Encysted					Activated					Breached					Excysted				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
15	76	50	61	96	100	24	50	39	4	-	-	-	-	-	-	-	-	-	-	-
30	46	37	19	75	100	54	61	55	25	-	-	2	26	-	-	-	-	-	-	-
45	17	23	13	72	96	33	38	13	28	4	33	26	34	-	-	17	13	40	-	-
60	14	13	7	57	91	37	27	16	43	9	4	16	24	-	-	45	44	53	-	-
90	7	3	2	11	96	18	13	22	89	4	11	-	-	-	-	64	84	76	-	-
120	2	-	2	8	96	2	3	2	92	4	-	3	-	-	-	96	94	96	-	-

(A) 0.5% trypsin and 0.5% bile salts; (B) 1% trypsin and 1% bile salts; (C) 1% trypsin and 0.5% bile salts; (D) 0.5% bile salt; (E) 0.5% trypsin in Earle's BSS.

The time lapse to reach excystment varied among the groups. Thus, the percentage of excystment increased for 45-120 min in parasites cultivated in medium A, B and C, reaching similar values at 120 min (94-96%) (Figure 1). Excysted metacercariae were successfully maintained for up to 180 min. After that, some worms presented an ovoid shape, absence of movement, suggesting the metacercariae were unviable. Metacercariae stained with trypan blue were revealed.
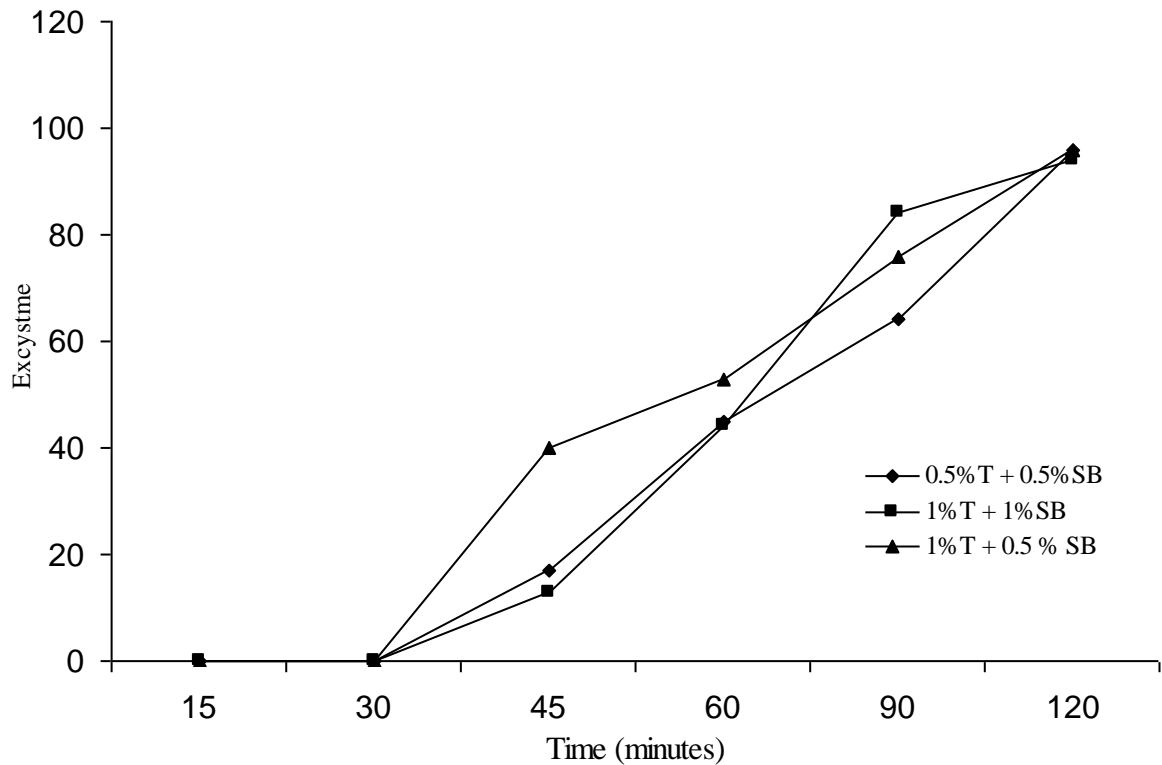
Figure 1. Percent excystation of *E. paraensei* in different concentrations of alkaline trypsin bile salts medium at 40 ºC for 15-120 minutes.

3.2. Morphological and morphometric findings

Microscopic examination of encysted metacercariae showed slow movements within the inner cyst. The activated stage was translucent with the outer layer of the cyst digested. This stage contained actively moving parasites within the cysts (data not shown). However, metacercariae maintained in medium D did not have a digested outer layer.

At the breached stage, the anterior end of metacercariae pushes a specific region of the cyst several times until it exits outside, while the end region stays inside the cyst. In the next step, this posterior region pushes the remaining body outside.

Fresh living excysted metacercariae analyzed by light microscopy showed muscular pharynx, ventral sucker and excretory concretions in the protonephridial tubules located in each side of the body (Figure 2A). The body was elongated and slender, slightly flattened dorsoventrally, enlarged at the anterior end and conical at the posterior end (Figure 2B). In those specimens, intensive movement of distention and contraction of the musculature between the oral sucker and ventral sucker and a truncated forebody were observed (Figure 2B). The posterior end of body contains marked conglomerate cells, which can be distinguished from parenchymal cells (Figure 2C).
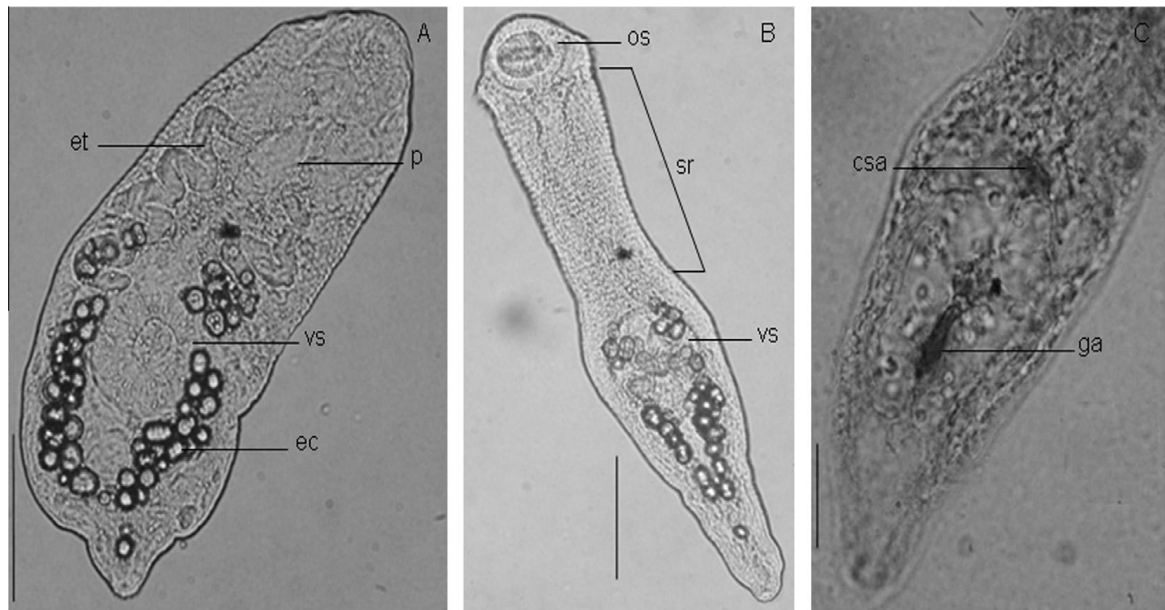
Figure 2. Light micrographs of excysted metacercariae of *E. paraensei*. (A) Excysted metacercariae after 2 hours showing pharynx [p], excretory tubule [et] containing excretory concretions [ec] and ventral sucker [vs]. (B) Excysted metacercariae after 2 hours showing striated region [sr] on the tegument between oral sucker [os] and ventral sucker [vs]. (C) Posterior end of excysted metacercariae stained with hydrochloric carmine showing genital anlage [ga] and cirrus sac anlage [csa]. Scale bars= 250μm appropriated to all figures.

Confocal images showed the oral sucker surrounded by the peristomic collar (head collar) with spines. The body surface presented ventral scales densely packed with similar squamous spines between the collar and the ventral sucker uniformly distributed (Figure 3A and 3B). The pharynx bulbar connects to the esophagus, which bifurcates immediately anterior at the level of the ventral sucker, forming two short ceca, which reach the posterior half of the body at the level of the undifferentiated testes and ovary (Figure 3C). The ventral sucker is situated below the middle of the body and is larger than the ventral sucker (Figure 3D).
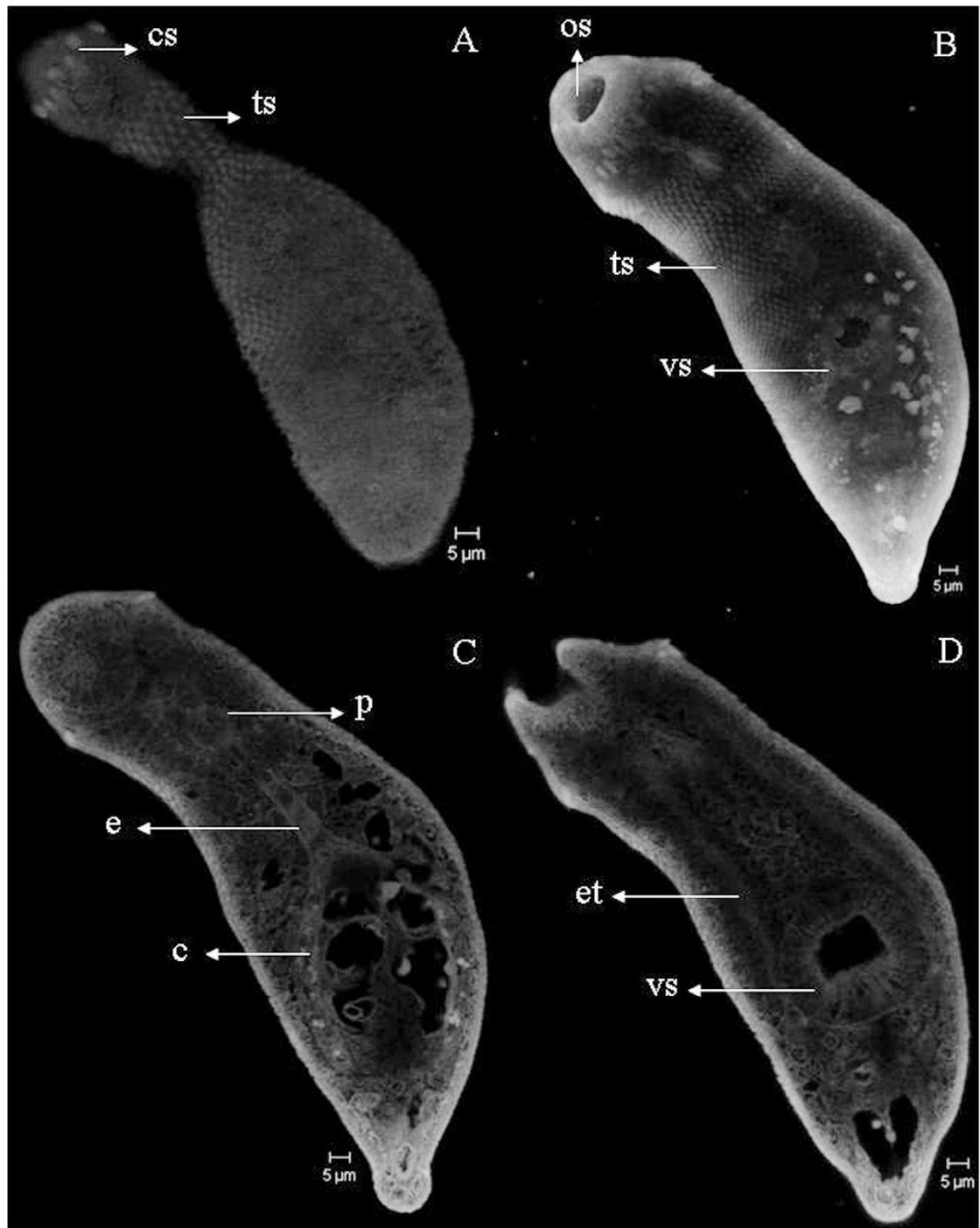
Figure 3. Confocal laser scanning microscopy images of whole-mounts preparations of excysted metacercariae of *Echinostoma paraensei* stained with hydrochloric carmine showing: (A) collar spine [cs] and tegumentar spines [ts]. (B) Oral sucker [os], ventral sucker [vs] and tegumentar spines [ts]. (C) Pharynx [p], esophagus [e] and ceca [c] and (D) Ventral sucker [vs] and excretory tubules [et].

The reproductive system is composed of a genital anlage, comprised of a set of opaque cells located under the ventral sucker in the posterior third of the body (Figure 4A). It is enveloped by a thick coat of cells which communicate through a column of cells toward the

undeveloped cirrus sac anterior to the ventral sucker and under the bifurcation of the esophagus (Figure 4B). The cirrus sac primordium is composed of a conglomerate of cells in which the testes and ovary anlage share similar morphology (Figure 4B). The calcareous corpuscles are distributed throughout the posterior region surrounding the ventral sucker and protonephridial tubules (Figure 4B).
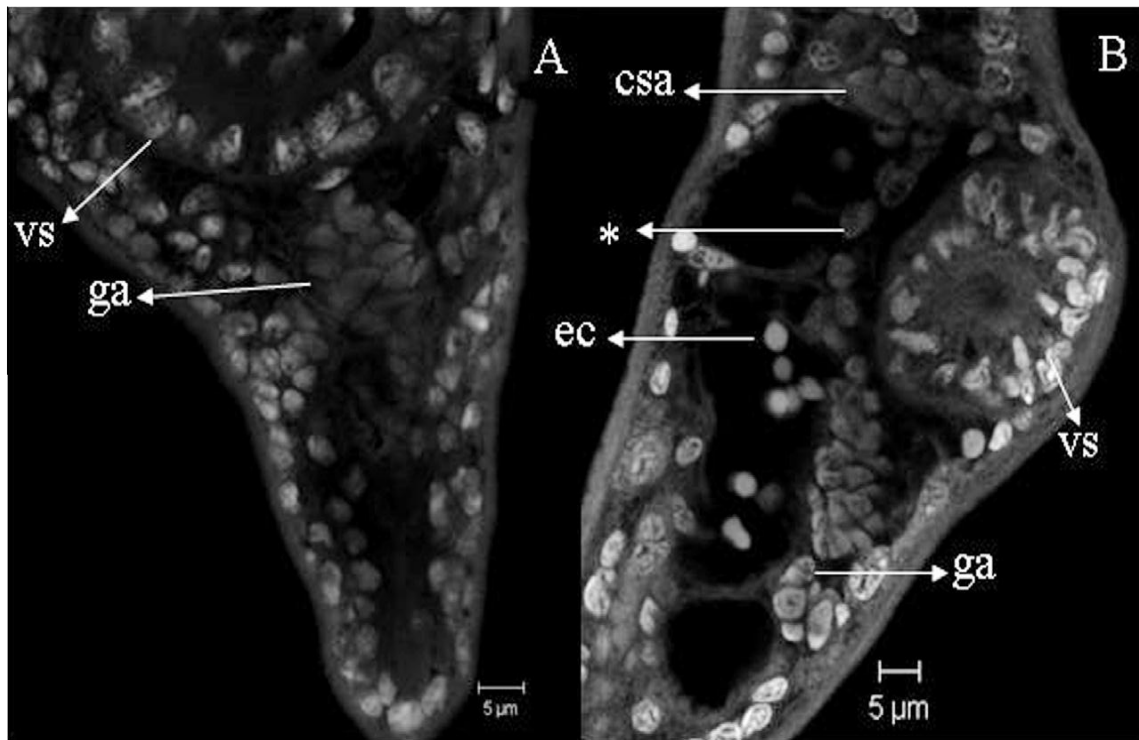
Figure 4. Confocal laser scanning microscopy images of whole-mounts preparations of posterior end of excysted metacercariae of *Echinostoma paraensei* stained with hydrochloric carmine showing (A) genital anlage [ga] of testes and ovary under to ventral sucker [vs] and (B) excretory concrection [ec] primordium genital [ga] of testes and ovary under to the ventral sucker [vs] connect to cirrus sac anlage [csa] by a column of cells [*].

Confocal images also revealed an outer cyst wall and the inner cyst wall disrupted after excystation (Fig. 5).
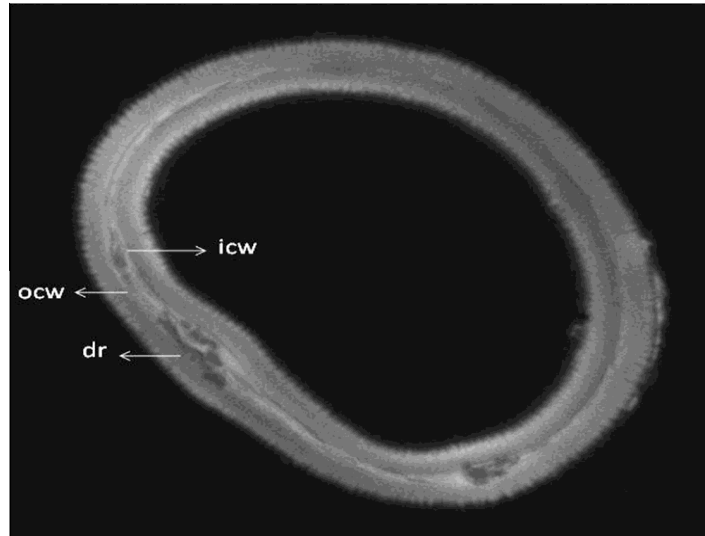
Figure 5. Confocal laser scanning microscopy images of whole-mounts preparations of metacercariae cyst of *Echinostoma paraensei* after excystation stained with hydrochloric carmine showing the outer cyst wall [ocw], the inner cyst wall [icw] and the digestion region between both wall [dr].

The measurements of juvenile metacercariae stained with hydrochloric carmine are summarized in Table 2.

Table 2. Morphometric data of excysted metacercariae of *Echinostoma paraensei*.

Parameter	Mean ± S.E.
Body length	173 ± 25
Body width	51 ± 11
Ratio 1 - Body length/ body width	3.5±0.9
Collar length	33 ±4
Collar width	45 ±10
Pharynx length	17 ± 3
Pharynx width	14 ± 5
Oral sucker length	31 ±3
Oral sucker width	28 ±4
Ventral sucker length	34 ± 6
Ventral sucker width	33 ± 6
Ratio 2 - Ventral sucker length/ oral sucker length	111 ± 16
Distance oral sucker to ventral sucker	80 ± 15
Distance between oral sucker to ventral sucker	52 ± 15
Distance ventral sucker to posterior end extremity	57 ± 10
Ratio 3 - Body length/ distance ventral sucker to posterior end extremity	305 ± 33
Excretory concretions	25±3.9

(mean ± S.E) All measures are in micrometers (n=25).

4. Discussion

Although excystation in *E. paraensei* is a known phenomenon, other stages remain unknown. Here we evaluated these stages through microscopy and morphometry. First, we demonstrated that metacercarial cysts obtained from *B. glabrata* snails are grape-shaped organisms covered with a very thick translucent outer layer and a refractive inner layer. Previous studies suggest that the outer layer originates from the host (Stein & Basch 1977; Ohyama 1998).

As the present studies illustrate, the time lapsed to reach excystment varied among the culture media. Although the onset of excystation started at 45 min, almost 100% of excystated stage was only reached after two hours. This result contrasts with studies performed in *E. caproni* (Ursone & Fried 1995; Fried & Reddy 1997), *E. lie* and *E. revolutum* (Fried & Emili 1988) and *E. trivolvis* (Smoluk & Fried 1994). In all of these species, the *in vitro* metacercarial development was faster. However, other studies using *E. paraensei* were partially successful in obtaining activated, breached and excysted metacercariae after two hours in culture (Fried & Reddy 1997).

This investigation demonstrated several phenotypic characteristics shared between *E. paraensei*, *E. trivolvis* and *E. caproni*: four morphological stages (encysted, activated, breached and excysted), slow movements or moving parasites within the cysts, disruption and excystation (Smoluk & Fried 1994; Ursone & Fried 1995).

The role of alkaline trypsin and bile salt during the steps of metacercarial development has been studied in several species of Digenea (see the review of Fried 1994). We evidenced that not all tested media allowed the development of breached and excysted stages, suggesting that both alkaline trypsin and bile salt play a role in the development of metacercariae (Fried 1994; Smoluk & Fried 1994). In this sense, we suggest that media containing only bile salt are patterns to study the activation stage of the metacercariae cyst.

Light microscopy of *E. trivolvis* has demonstrated that breached stage exits partially from the inner cyst via an opening in the inner cyst (Smoluk & Fried 1994). Our results not only confirm this finding, but add new morphological data for *E. paraensei*. Confocal images detailed the outer and inner layer of the cyst and evidenced the mechanism by which metacercariae leave the cyst. The anterior end of the metacercaria pushes a specific region of the cyst several times until it exits outside, while the end region stays inside the cyst. In the next step, this posterior region pushes the remaining body outside. It is possible that both the outer and inner layers are digested by trypsin, while bile salts stimulate larval movement (Ohyama 1998). On the other hand, *Clonorchis sinensis* excystation requires acid pepsin and bile salt (Ohyama 1998). After excystation, the wall of free cysts had layers as previously reported for *E. paraensei* (Lie & Basch 1967; Stein & Basch 1977), as well as plugs, previously described for *E. trivolvis* (Smoluk & Fried 1994).

As determined by confocal microscopy, juvenile metacercariae presented excretory concretions (calcareous corpuscles) with distribution throughout the excretory tubules, as also observed in *E. trivolvis* and *E. caproni* (Saxton et al. 2008). Previous studies have shown that calcareous corpuscles are of cellular origin (Pawlowski et al. 1988; Xifeng & Baodong 1998; Vargas-Parada et al. 1999a), have different sizes (Vargas-Parada et al. 1999b) and are irregularly spherical or ovoid in shape (Smith & Richards 1993). These descriptions are in line with our findings for *E. paraensei*.

Because excretory concretions are located in several places in metacercariae, there are conflicting explanations for their role (Xifeng & Baodong 1998; Vargas-Parada et al. 1999ab; Yang 2004; Martínez et al. 2005; Khalifa et al. 2011; Chalar et al. 2013). Calcareous corpuscles are believed to be a principal source of calcium carbonate, magnesium and phosphate (Smyth 1969; Smith & Richards 1993), with a possible role in excretory activity (Vargas-Parada et al. 1999b), osmoregulation (Mackiewicz & Ehrenpris 1980), metabolic

neutralization of acidic products (Slais 1973) and protection against calcification (Desses 1963).

In the present study, we also observed excretory concretions surrounding the ventral sucker, which suggests a relationship with motor activity, such as energy transformation, because calcareous corpuscles store ions and can act as an energy source (Rodrigues et al. 1997). Our experiment and published studies of the *E. caproni* model (Fried & Reddy 2000) show that excretory concretions have tendency to disappear over time. We hypothesize that excretory concretions are not lost, instead becoming less visible after emptying their contents. It is also worth pointing out that the excretory concretions may play a role in morphogenesis, because they are located close to the genital anlage, which gives rise to the reproductive system (Souza et al. 2011). It can be also hypothesized that the calcareous corpuscles are involved in parasite differentiation and growth (Rodrigues et al. 1997).

In the present study, *E. paraensei* showed lesser number than *E. caproni* and *E. trivolvis* (Saxton et al. 2008), while the number was similar to that found in other studies of *E. paraensei* (Fried and Reddy 1997). Our results confirm that calcareous corpuscles show quantitative variations in parasites of these taxa.

To date, morphometry has been used to establish morphological features of excysted metacercariae of *E. caproni* and *E. paraensei* (Fried & Reddy 1997). Our morphometric data were smaller in value than reported in previous studies of the same species studied here (Fried & Reddy 1997). Although this issue has never been studied in metacercariae, previous results from adult worms demonstrate intraspecific variability between isolates from different regions (Maldonado et al. 2005). We suggest that such variation may be related to the characteristics of each isolate of *E. paraensei*. However, future studies are needed to confirm this hypothesis. In addition, we present other new morphometric parameters, which should be important for comparison among species of *Echinostoma* based on allometric study (Valero et al. 2001).

After the first description of *E. paraensei* four decades ago (Lie and Basch 1967), the morphology of adult worms, eggs, miracidia and rediae of *E. paraensei* has been analyzed by both light and scanning electron microscopy (Fujino et al. 2000; Maldonado et al. 2001; Pinheiro et al. 2004ab; Ferraz et al. 2012; Souza et al. 2011). To date, the morphological characteristics of excysted metacercaria from *E. paraensei* have not received sufficient study. For example, there is no information on the use of light and confocal microscopy. Most importantly, we noted that structures that are apparently absent under light microscopy are revealed by scanning laser confocal microscopy. Due to its better resolution, confocal laser scanning microscopy, using a reflected mode with tomographic sections, allows new interpretations, modifying the current descriptions of the internal morphological structure in parasitic trematodes (Neves et al. 2005). From the biological standpoint, our data provide detailed information of the development kinetic of the reproductive system, from the pre-ovigerous to ovigerous stage (Souza et al. 2011).

In short, trypsin and bile salts associated are fundamental for the *in vitro* metacercarial excystation of *E. paraensei*. This is the first detailed information of all stages of metacercarial excystation through light and confocal microscopy.

Acknowledgements

References

Chalar C, Salomé M, Señorale-Pose M, Marin M, Williams C.T., Dauphin Y. 2013. A high resolution analysis of the structure and chemical composition of the calcareous corpuscles from *Mesocestoides corti*. Micron. 44:185-92.

Desses S.S. 1963. Calcium accumulation in larval *Echinoccus multilocularis*. Can J Zool 41: 1055-1059.

Ferraz J, Souza J., Costa-Silva M, Torres E. Santana A, Lanfredi R, Maldonado A, Garcia J. 2012. Effect of praziquantel on adult *Echinostoma paraensei* worms in experimentally infected mice. Parasitol. Res. 111:143-148.

Fried B, Emili S. 1988. Excystation *in vitro* of *Echinostoma liei* and *E. revolutum* (Trematoda) metacercariae. J. Parasitol. 74:98-102.

Fried B. 1994. Metacercarial excystment of trematodes. Adv. Parasitol. *33*:91-144.

Fried B, Huffman J.E. 1996. The biology of the intestinal trematode *Echinostoma caproni*. Adv. Parasitol. 38: 311-368.

Fried B, Reddy A. 1997. Comparative studies on excystation and morphological features of the metacercariae of *Echinostoma paraensei* and *E. caproni*. Int. J. Parasitol. 27:889-901.

Fried B, Reddy A. 2000. Postmetacercarial changes in *Echinostoma caproni* maintained in a defined medium plus calf serum. Korean J. Parasitol. 38:173-175.

Fried B, Schneck J.L., Ponder E.L. 2004. Effect of tonicity, digestive enzymes and bile salts, and nutrient media on the survival of excysted metacercariae of *Echinostoma caproni*. Parasitol. Res. 93: 527-530.

Fried B, Peoples R.C. 2007. Viability of the encysted metacercariae of *Echinostoma caproni* judged by light microscopy versus chemical excystation. J Parasitol 93:947.

Fried B, Roth R.M. 1974. *In vitro* excystment of the metacercariae of *Parorchis acanthus*. J. Parasitol. 60:465.

Fried B, Schmidt K.A., Sorensen R.E. 1997. *In vivo* and ectopic encystment of *Echinostoma revolutum* and chemical excystation of the metacercariae. J. Parasitol. 83:251-254.

Irwin S.W.B., McKerr G, Judge B.C., Moran I. 1984. Studies on metacercarial excystment in *Himasthla leptosoma* (Trematoda: Echinostomatidae) and newly emerged metacercariae. Int. J. Parasitol. 4:415-421.

Khalifa R.M., Mazen N.A., Marawan A.M., Thabit H.T. 2011. Histochemical and ultrastructural studies on the calcareous corpuscles and eggs of *Taenia taeniaeformis* and *Dipylidium caninum*. J. Egypt. Soc. Parasitol. 41:513-28.

Lie K.J., Basch P.F. 1967. The life history of *Echinostoma paraensei* sp.n. (Trematoda:Echinostomatidae). J. Parasitol. 53:1192-1199

Mackiewicz J.S., Ehrenpris M.B. 1980. Calcareous corpuscles distribution in caryophillid cestode: possible evidence of cryptic segmentation. Proc. Helminthol. Soc. Wash. 47:1-9.

Maldonado A. Jr., Vieira G.O., Garcia J.S., Rey L., Lanfredi R.M. 2001. Biological aspects of a new isolate of *Echinostoma paraensei* (Trematoda: Echinostomatidae): susceptibility of sympatric snail and the natural vertebrate host. Parasitol. Res. 87: 853-859.

Maldonado A. Jr., Zeitone B.K., Amado L.A., Rosa I.F., Machado-Silva J.R., Lanfredi R.M. 2005. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. J. Helminthol. 79: 345-351.

Maldonado A. Jr, Lanfredi R.M. 2009. Echinostomes in the wild. In: Fried B; Toledo R. (Eds.), The biology of Echinostomes from the molecule to the community. Springer, New York, pp. 129-145.

Martínez C., Paredes R., Stock R.P., Saralegui A., Andreu M., Cabezón C., Ehrlich R., Galanti N. 2005. Cellular organization and appearance of differentiated structures in developing stages of the parasitic platyhelminth *Echinococcus granulosus*. J. Cell. Biochem. 94:327-35.

Neves R.H., Pereira M.J.S., Gomes D.C., Oliveira R.M.F., Machado-Silva J.R. 1998. Morphometric differences of adult worms from sympatric samples of *Schistosoma mansoni* Sambon, 1907 isolated from rodents and humans. Mem. Inst. Oswaldo. Cruz. 93: 309-312.

Neves R.H., Biolchini C.L., Machado-Silva J.R., Carvalho J.J., Branquinho T.B., Lenzi H.L., Hulstijn M., Gomes D.C. 2005. A new description of the reproductive system of *Schistosoma mansoni* (Trematoda: Schistosomatidae) analyzed by confocal laser scanning microscopy. Parasitol. Res. 95:43-49.

Ohyama F., 1998. Effect of acid pepsin pretreatment, bile acids and reductants on the excystation of Clonorchis sinensis (Trematoda: Opisthorchiidae) metacercariae *in vitro*. Parasitol. Int. 47:29-39.

Pawlowski I.D., Yap K.W., Thompson R.C. 1988. Observations on the possible origin, formation and structure of calcareous corpuscles in taeniid cestodes. Parasitol. Res.74:293-296.

Pinheiro J., Maldonado A., Lanfredi R.M. 2004a. Light and scanning electron microscopy of the miracidium of *Echinostoma paraensei* (Trematoda, Echinostomatidae). Vet. Parasitol. 121:265-275.

Pinheiro J., Maldonado A., Attias M., Lanfredi R.M. 2004b. Morphology of the rediae of *Echinostoma paraensei* (Trematoda: Echinostomatidae) from its intermediate host *Lymnaea columella* (Mollusca, Gastropoda). Parasitol. Res. 93:171-177.

Rodrigues J.J., Ferreira H.B., Farias S.E., Zaha A. 1997. A protein with a novel calcium-binding domain associated with calcareous corpuscles in *Echinococcus granulosus*. Biochem. Biophys. Res. Commun. 237:451-456.

Saxton T.M., Fried B., Peoples R.C. 2008. Excystation of the metacercariae of *Echinostoma trivolvis* and *Echinostoma caproni* in a trypsin-bile salts-cysteine medium and morphometric analisys of the larvae. J. Parasitol. 94:669-671.

Slais J. 1973. Functional morphology of cestode larvae In: Dawnes B (ed). Adv. Parasitol. Academic Press, New York 396-466.

Smith S.A., Richards K.S. 1993. Utrastructure and microanalyses of the calcareous corpusclesof the protoscolices of *Echinococcus granulosus*. Parasitol. Res. 79:245-250.

Smoluk J.L and Fried B. 1994. Light microscopy observation of *Echinostoma revolutum* (Trematoda) metacercariae during *in vitro* excystation. Parasitol. Res. 80: 435-438.

Smyth J.D. 1969. The physiology of cestodes. Freeman and Company. San Francisco

Souza J.G.R., Garcia J.S., Manso P.P.A., Neves R.H., Maldonado A, Machado-Silva J.R. 2011. Development of the reproductive system of the *Echinostoma paraensei* in *Mesocricetus auratus* analyzed by light and confocal scanning laser microscopy. Exp. Parasitol. 128:341-346.

Stein P.C., Basch P.F. 1977. Metacercarial cyst formation *in vitro* of *Echinostoma paraensei*. J. Parasitol. 63: 1031-1040.

Ursone R.L., Fried B. 1995. Light microscopy observation of *Echinostoma caproni* metacercariae during *in vitro* excystation. J. Parasitol. 69: 253-257.

Valero M.A., Darce N.A., Panova M., Mas-Coma S. 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. Vet. Parasitol. 102 (1-2):85-100.

Vargas-Parada L., Lanclette J.P. 1999. Role of the calcareous corpuscles in cestode physiology: a review. Rev. Latinoam. Microbiol. 41:303-307.

Vargas-Parada L., Merchant M.T., Willms K., Laclette J.P. 1999. Formation of calcareous corpuscles in the lumen of excretory canals of *Taenia solium* cysticerci. Parasitol. Res. 85:88-92

Yang H.J. 2004. Immunoblot findings of calcareous corpuscles binding proteins in cyst fluid of *Taenia solium* metacestodes. Korean. J. Parasito. 42:141-143.

Xifeng T., Baodong Z. 1998. Ultrastructural observation on the formation and metabolism of calcareous corpuscles in *Cysticercus cellulosae*. Chin. J. Parasitol. Parasitic. Diseases. 16:364-367.

4 *IN VITRO* ACTIVITY OF ARTESUNATE AGAINST NEWLY EXCYSTED METACERCARIAE OF *ECHINOSTOMA PARAENSEI* (TREMATODA: DIGENEA)

Joyce G.R. Souza[1], Eduardo J. Lopes Torres[1], Juberlan S.Garcia[2], Rosângela Rodrigues-Silva[3], Arnaldo Maldonado Junior[2] and José Roberto Machado-Silva[1]

[1] Laboratório de Helmintologia Romero Lascasas Porto, Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Centro Biomédico, Universidade do Estado do Rio de Janeiro, Av. Prof. Manoel de Abreu 444/5º andar, Vila Isabel, 20511-070, Rio de Janeiro, Brazil.

[2] Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, 21040-360, Rio Janeiro, Brazil.

[3] Laboratório de Helmintos Parasitos de Vertebrados, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, 21040-360, Rio Janeiro, Brazil.

Corresponding author: Tel: (+55 21 2868-8048); E-mail: machado@uerj.br

Abstract

Background: Chemotherapy of food-borne trematodes relies on two drugs, praziquantel and tricabendazole. However, due to resistance to these drugs, there is growing interest in finding alternative therapies. Traditional Chinese medicine has long used plant oil extracts as sources of bioactive compounds with antiparasitic activity. Even though artemisinin derivatives have demonstrated a broad spectrum of activity against adult trematodes, their activity against larval stages is not well understood. Species of the genus *Echinostoma* are used as models to test effective compounds against food-borne trematodes. This study evaluated the

anthelmintic activity of crude artesunate extracts *in vitro* on newly excysted metacercariae of *E. paraensei*.

Methods: The flukes were incubated with 1µg/mL, 10µg/mL, 25µg/mL, 50µg/mL and 100µg/mL of artesunate for 4, 12, 24, 48 and 72 h. Microscopic assessment of motility (lively movement or slow movement), morphological changes (damaged tegument) and death of worms was carried out.

Results: Incubation for short periods and administration of low doses did not cause any change in motility or morphology. When the exposure time and concentration of artesunate increased, there were changes in motor activity, tegument damage and death. Images taken by inverted microscope showed no changes 4 h after exposure. However, 100µg/mL and 50µg/mL caused deleterious effects at 24 and 48 h, respectively. Images taken by scanning electron microscopy of helminths incubated with 50 and 100µg/mL showed that artesunate caused swelling, blebs, holes, eruption, furrowing, low collar spines and low tegument spines.

Conclusions: The results showed that (i) motility and tegument changes are both time and dose dependent; and (ii) *in vitro* models using excysted metacercariae are advantageous for drug testing because they are cheap and easy to obtain, avoiding the unnecessary death of the vertebrate host. Furthermore, this is the first report of *in vitro* anthelmintic activity of artesunate against newly excysted metacercariae.

Keywords: *Echinostoma paraensei*; artesunate; *in vitro*; motor activity; morphological alterations; mortality; scanning electron microscopy.

Background

Parasitic flukes are responsible for human and livestock infections predominantly in developing countries [1]. Liver, lung and intestinal flukes are responsible for causing food-borne trematodiasis [2], which is an emerging public health problem, notably in the southern

Asia and the western Pacific [3]. Despite increased support for research into treatment and morbidity control in recent decades, praziquantel (PZQ) and tricabendazole (TCZ) are still the only available drugs [4]. PZQ is effective against *Clonorchis sinensis* (Kin and Min, 1998), *Opistorchis viverrini* [6], *Echinostoma paraensei* and *E. caproni* [7, 8, 9] and *Paragonimus westermani* [10]. PZQ causes rapid contraction of the worm musculature, leading to a loss of worm movement, and rapid bleb formation and vacuolization of the tegument [11]. In turn, TCZ is the current mainstay drug for control of fasciolosis, because it is safe and effective against both juvenile and adult flukes [12].

Unfortunately, decreased drug sensitivity or resistance to antiparasitic drugs has been documented [13, 14, 15], a situation that poses a challenge to a challenge for the chemotherapy of helminthiasis [16]. In an effort to overcome this issue, novel chemicals with high antiparasitic activity potential have been assayed [17]. In this context, artemisinin-based therapy (artemether, artesunate and dihydroartemisinin) have displayed significant activity against adult flukes, such as *Clonorchis sinensis* [18], *Fasciola hepatica* [12, 19, 20, 21], *Echinostoma caproni* [4] and *Schistosoma mansoni* [22]. Interestingly, immature stages are more susceptible to antiparasitic compounds than adult worms [15, 23].

Rearing of *E. paraensei* in the laboratory is easy, cheap and provides a large number of larval stages, allowing comprehensive research into echinostomes and echinostomiasis [24]. Our earlier studies evidenced the main steps for *in vitro* excystation of *E. paraensei*. The highest ratio was achieved with exposure to trypsin and bile salts approximately two hours after incubation [25]. This opens a new line of investigation, in which the potential of pharmaceuticals with anthelmintic activity can be evaluated using *in vitro* cultured juvenile parasites [26]. This possibility is especially important because it avoids unnecessary killing of the experimental host during the preliminary stage of drug screening and contributes to the 3R principles: reduce, replace and refine [8].

The aims of this study were to investigate the *in vitro* activity of artesunate on newly excysted *E. paraensei* metacercariae and to describe drug-induced alterations by light and scanning electron microscopy.

Methods

Ethics statement

All experiments involving hamsters were been performed in accordance with the rules of the Committee on Ethical Use of Animals of Oswaldo Cruz Foundation (permit CEUA LW-051/14).

Host-parasite model

The life cycle was maintained at the Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios IOC-Fiocruz, Rio de Janeiro (Brazil), as described by Garcia et al. [27]. Briefly, *Mesocricetus auratus* (golden hamster) were exposed to metacercariae derived from experimentally infected *Biomphalaria glabrata* snails infected with *E. paraensei* (Sumidouro isolate). Metacercarial cysts were obtained by dissection of the pericardium region of *B. glabrata* snails under a stereomicroscope. Metacercariae were grape-shaped organisms covered with a very thick translucent outer layer and a refractive inner layer. The cysts were washed in Locke's solution (9 g/l NaCl, 0.4 g/l KCl, 0.2 g/l CacCl2, 0.2 g/l NaHCO3) at room temperature and immediately used.

Excystation

The *in vitro* excystation procedure used was similar to our previous studies [25]. Briefly, cysts were treated with 0.5% trypsin (Sigma) and 0.5% bile salts (Sigma) dissolved in Earle's BSS (6.8 g/l NaCl, 0.4 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l $MgSO_4$, 0.125 g/l $NaH_2PO_4.H_2O$, 2.2 g/l $NaHCO_3$ and 1 g/l D-glucose). Twenty metacercarial cysts were used for each tested

solution in 3.5-cm Petri dishes, in a total volume of 3 mL of fresh trypsin bile salt medium at 37 ºC and 5% CO_2 atmosphere, pH=8.0±0.2, with 7.5% $NaHCO_3$.

Drug

Artesunate was obtained from Oswaldo Cruz Foundation (Fiocruz/RJ, Brazil). The drug was initially prepared as stock solution with 10 mg of artesunate in 0.6 mL in the solvent dimethyl sulfoxide (DMSO) (Sigma) added to 0.4 mL of Earle's BSS. The concentration of artesunate in this study was based on previous studies [4].

In vitro **assay**

The excysted worms were immediately washed three times in Locke's solution. Then the worms (n=10/plate) were in culture medium (RPMI 1640 supplemented with 10% bovine fetal serum, 200 µg/mL streptomycin, 200 U/mL penicillin and 0.25 µg/mL Amphoterecin B, Sigma, USA) in 6 well plates (4 mL/plate). The experiments were performed in a biological safety cabinet (Telstar Technologies, Bio II A/P, Spain). Then the flukes were incubated with different concentrations of artesunate (1µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL) for the designed time intervals (4, 12, 24, 48 and 72 h). Flukes incubated with the solvent (0.6% DMSO) alone were used as drug-free controls. The experiments were performed in triplicate and the cultures were incubated at 37 °C under a 5% CO_2 atmosphere in a Sanyo InCuSafe incubator (MCO-17AIC, USA).

Assessment of drug effect

Images of the morphological features were taken by a digital camera (Bell, 3.0 MP Japan) attached to an inverted microscope (Nikon Eclipse TS100-F, Japan).

The effect of artesunate on each fluke was determined by 2 min of examination. A detailed description of how worms were scored can be found in Panic et al. (2013). Briefly, the scoring was dead worms (0), worms with impaired movement and marked tegument

damage (1), slow movement or notable damage tto tegument (2) and lively movement and undamaged tegument (3). The activity of artesunate was assessed by comparing the number of affected worms in any treatment group with that corresponding to the control group.

Examination by scanning electron microscopy

E. paraensei excysted larvae cultured for 24 and 48 h were prepared for conventional scanning electron microscopic examination as previously described [28]. Briefly, they were washed in PBS at pH=7.4, fixed in Karnovsky's solution for 24 h at 4 ℃, dehydrated in an increasing ethanol series (30%, 50%, 70%, 80%, 90% and absolute), for 15 min at each concentration, and then point dried in a Blazers critical point dryer Bal-Tec CPD-030. The samples were submitted to metallization with gold in a layer approximately 20-25 nm thick and then observed under an FEI Quanta 250 scanning electron microscope (CENABIO - Microscopy Division).

Statistical analysis

One-way analysis of variance (ANOVA) and the post-hoc Tukey test were used to compare the mean differences of the excystment rate for each medium tested. Statistical significance was taken as $P < 0.05$.

Results

Assessment of drug effect

The effect of artesunate on newly excysted metacercariae is shown in Figure 1 and Table 1. The negative control parasites remained viable throughout the observation period. The results showed both time and drug concentration-dependent effects on parasites. After incubation for 4 h, no artesunate concentration induced change on the parasites' viability, since all examined worms showed lively movement similar to those in the drug-free control group (Figure 1). At lower artesunate concentrations (1 µg/mL and 10 µg/mL), viability was

not affected throughout the analysis period (Figure 1). At 25 µg/mL, artesunate had a deleterious effect on viability of *E. paraensei* after 48 h of incubation. However, viability was drastically reduced with increased artesunate concentration with the highest effect at 100 µg/mL or 50 µg/mL after 24 h or 48 h, respectively (Figure 1).

Worms exposed to different concentrations of artesunate were classified into four categories according to their activity or tegument organization (Table 1). After 48 h incubation, only slow movement or slow movement and damaged tegument were seen with 1 µg/mL and 10 µg/mL concentrations (Table 1). In contrast, all parasites exposed to 25 µg/mL showed intermediate performance leading to slow movement after 12-48 h incubation, slow movement and damaged tegument after 48 h, and few surviving worms after 72 h of incubation.

All worms exposed to 50 µg/mL of artesunate showed slow movement and damaged tegument after 12 h of incubation (Table 1). A more complex time-dependent effect was observed in the case of worms exposed for 24 h, with slow movement/damage tegument and death in almost equal proportions. After the longest interval (72 h), all worms were dead (Table 1).

The number of dead metacercariae increased with the drug exposure time. Not all worms exposed to the 100 µg/mL concentration were dead after 12 h of incubation, whereas all subsequent examinations (24, 48 and 72 h) revealed dead worms (Table 1).

Figure 1- Viability of newly excysted metacercariae of *Echinostoma paraensei* during *in vitro* incubation of RPMI 1640 plus different concentration of artesunate (ATS).
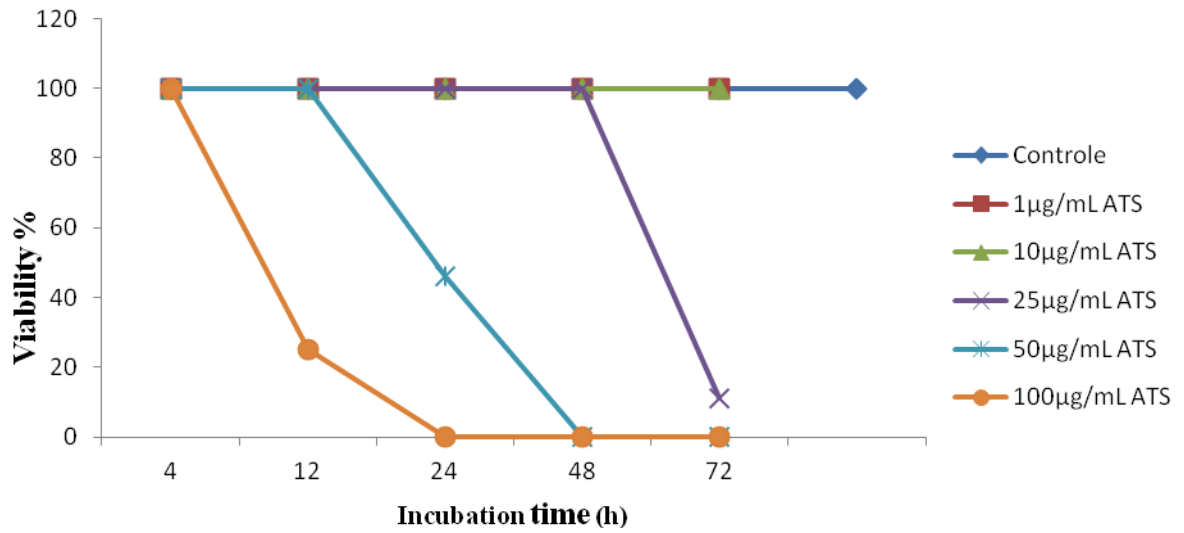
Table 1- Effect of different concentrations of artesunate (ATS) on newly excysted metacercariae of *Echinostoma paraensei* during *in vitro* incubation.

Time	ATS concentration	Lively movement	Slow movement	Slow movement and damage tegument	Dead
4 hours	Control	100	-	-	-
	1µg/mL	100	-	-	-
	10 µg/mL	100	-	-	-
	25 µg/mL	100	-	-	-
	50 µg/mL	100	-	-	-
	100 µg/mL	100	-	-	-
12 hours	Control	100	-	-	-
	1µg/mL	100	-	-	-
	10 µg/mL	100	-	-	-
	25 µg/mL	-	96	4	-
	50 µg/mL	-	-	100	
	100 µg/mL	-	-	25	75
24 hours	Control	100	-	-	-
	1µg/mL	100	-	-	-
	10 µg/mL	100	-	-	-
	25 µg/mL	-	100	-	-
	50 µg/mL	-	-	46	54
	100 µg/mL	-	-	-	100
48 hours	Control	100	-	-	-
	1µg/mL	-	84	16	-
	10 µg/mL	-	92	8	-
	25 µg/mL	-	89	19	-
	50 µg/mL	-	-	-	100
	100 µg/mL	-	-	-	100
72 horas	Control	100	-	-	-
	1µg/mL	-	80	20	-
	10 µg/mL	-	23,5	76,5	-
	25 µg/mL	-	-	10,5	89,5
	50 µg/mL	-	-	-	100
	100 µg/mL	-	-	-	100

The worms of the control group and those incubated in different concentrations of artesunate after the first 4 h showed similar morphology. The flukes were actively mobile with whole body movement of extension and contraction in the longitudinal axis, including oral and ventral sucker movement. They were translucent, with calcareous corpuscles in the excretory tubules (Figure 2A). The worms with slow movement after exposure to 25 μg/mL were translucent with the posterior body region paralyzed, narrowed and somewhat curved ventrally. The forebody (region anterior to the ventral sucker) was contracted more time than in the control group. There is low number of calcareous corpuscles in the excretory tubules, which were enlarged, mainly next to the excretory pore (Figure 2B). Worms exposed to higher concentrations showed reduced movement and tissue damages. In general, worms showed opaque yellowish color and undifferentiated internal organs. The parenchyma was diffuse and vacuolated, leaf shaped with shrunken and wider body than in the control group (Figure 2C). At this moment, minimal movement in the forebody indicated the worm was not dead (data not shown). Dead worms were characterized by their dark appearance, atypical body shape, highly vacuolated parenchyma, undifferentiated internal organs and calcareous corpuscles (Figure 2D).
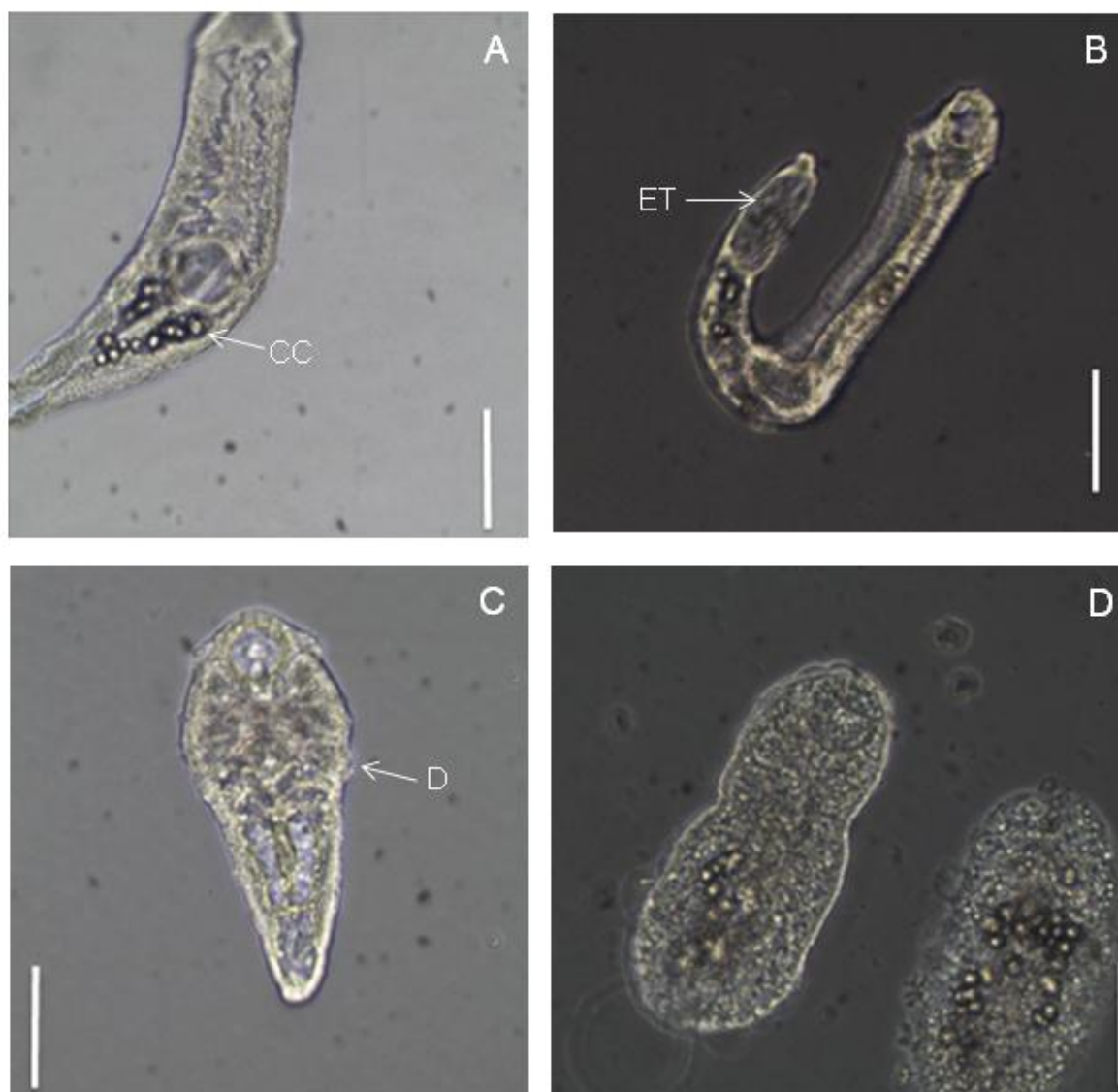
Figure 2- Light microscopy of newly excysted larvae of *Echinostoma paraensei* after incubation in different concentrations of artesunate. (A) Control group after 4 hours post incubated with some calcareous corpuscles [CC]. (B) 25µg/mL after 12h post-incubated showing tapered posterior region of body curved ventrally and dilated excretory tubules [ET]. (C) 50µg/mL at 12h post-incubation showing diffuse parenchyma and damage [D]. (D) 100µg/mL at 12h post-incubation showing the morphology of death larvae with several blebs. All scale bars 500µm.

Using the scanning electron microscopy (SEM) was possible to characterize the surface topography of these trematodes. Newly excysted metacercariae cultivated *in vitro* from the control group showed elongated and dorso-ventrally flattened body that was wider in the anterior ventral sucker region. The ventral sucker was located in the middle body and narrowed hind body (region between the ventral sucker and the posterior end) (Figure 3A).

The tegument of both the oral and ventral sucker was smooth (Figure 3A). The anterior end had a ventro-subterminal oral sucker and spherical papillae, located between the oral sucker and collar spines, which were conspicuous, slightly curved and pointed (Figure 3B-Insert). Next to the collar spines, a single or cluster of uniciliated and dome-shaped papillae were found (Figure 3C). The tegument is armed with rows of triangular scale-like spines, whereas spherical papillae are distributed either below or above the collar spines in the lateral part of the body (Figure 3D).

Samples exposed to artesunate analyzed using SEM showed several tegument changes which were related to different times of exposure. Artesunate provoked changes in the different regions of body surface: anterior, middle and posterior parts. Worms incubated with 100 µg/mL artesunate for 24 h showed changes in the anterior end, characterized by swelling. The collar spines and papillae were not affected, however in the ventral surface of the tegument, on the region between suckers displayed tegument with disordered spines, blebs and small holes (Figure 4A). Worms exposed for a longer time (48 h) to a lower dosage (50 µg/mL) showed similar lesions, but more intense. The oral sucker and surrounding tissue showed high swelling and papillae deformity, whereas unaltered spines in the region between suckers were found (Figure 4B). The region posterior to the ventral sucker showed some tegument swelling, which did not harbor small spines (Figure 4C) in helminthes incubated with 50 µg/mL for 48 h. The collar of spines showed empty pockets without dorsal spines. Blebs between suckers were also evidenced (Figure 4D). Some specimens showed folded tegument with subtegumental tissue exposure (Figure 4E) after exposure to the same concentration.
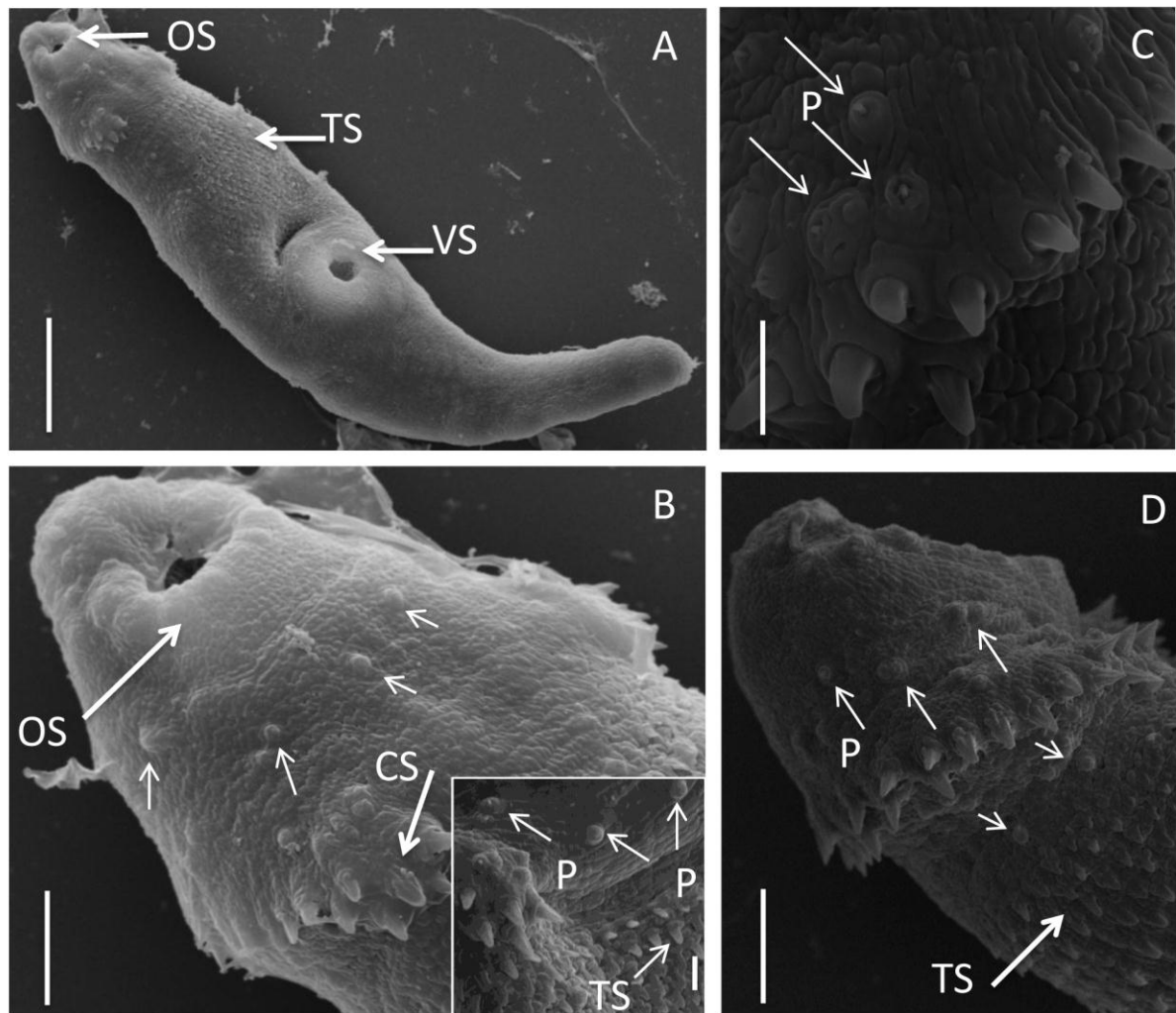
Figure 3- Scanning electron microscopy images of newly excysted metacercariae of *Echinostoma paraensei* for control group after *in vitro* incubation showing (A) General morphology of ventral view with oral sucker [OS], tegumental spines [TS], ventral sucker [VS] and narrow region posterior to ventral sucker; Scale bar: 20µm. (B) Anterior end of body showing oral sucker [OS] surrounded by collar spines with corner spines [CS] and papillae [arrow] in the region between oral sucker and collar spines (detail); Scale bar: 8µm. (C) Lateral view of a cluster of ciliated papillae [arrow] above corner spines; Scale bar: 2µm. (D) Lateral view of anterior end of body showing papillae under and above collar spines [arrow] and tegumental spines [TS] in the region between collar spines and ventral sucker. Scale bar: 100µm
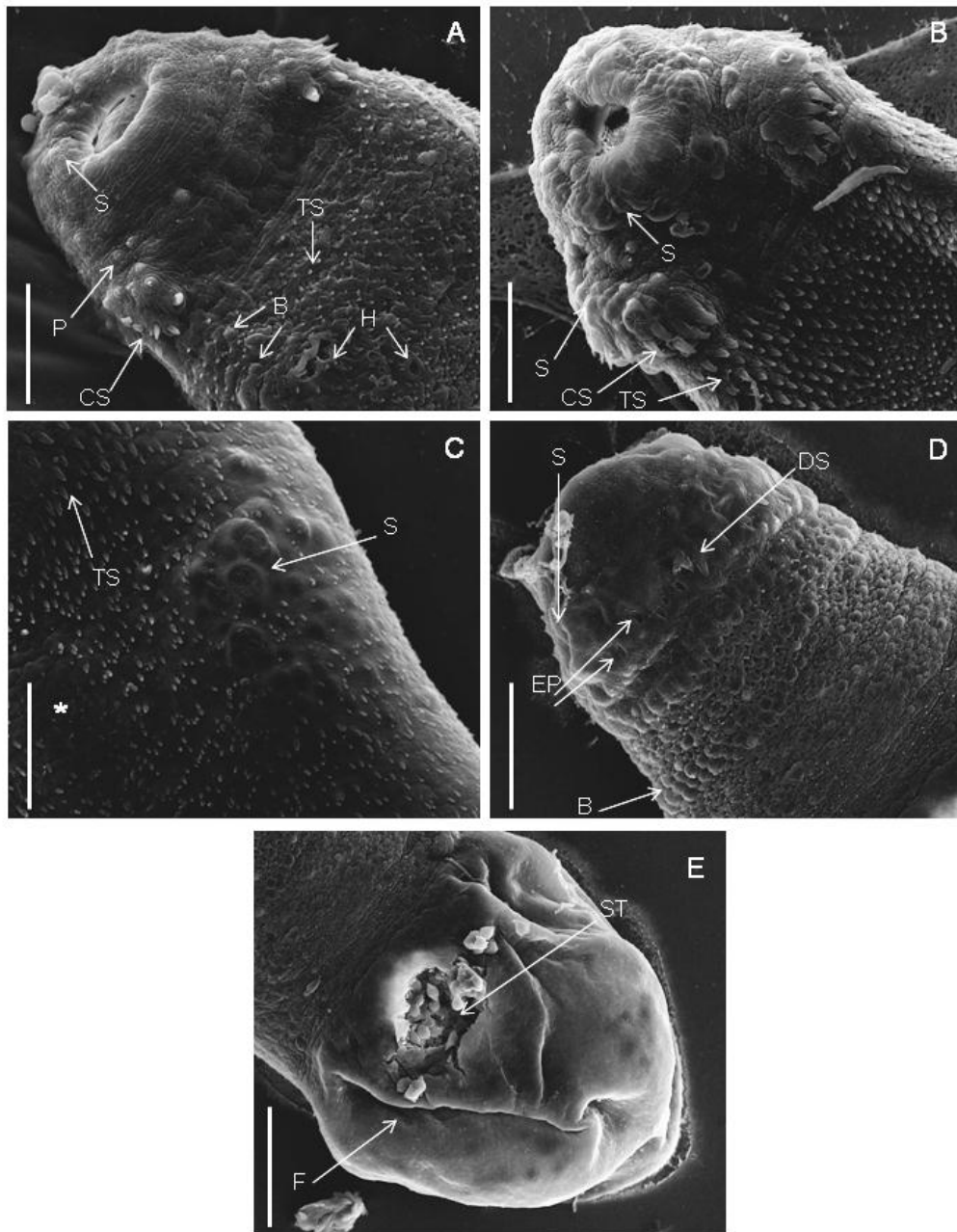
Figure 4- Scanning electron microscopy images of newly excysted metacercariae of *Echinostoma paraensei* after *in vitro* incubation with different concentration of artesunate. (A)- Anterior end of body of *E. paraensei* incubated with 100 µg/mL artesunate for 24h showing collar spines [CS], papillae [P], light swelling [S] and tegument between suckers with not uniform tegumental spines [TS], blebs [B] and holes [H]. Scale bar: 15µm. (B)- Anterior end of body of *E. paraensei* incubated with 50 µg/mL artesunate for 48h showing intense deformity and swelling [S], uniform tegumental spines [TS] and full corner spines [CS]. Scale bar: 15µm. (C)- Lateroventral view of posterior region of *E. paraensei* incubated with 50 µg/mL artesunate for 48h showing swelling [S] and low tegumental spines [*] [TS]. Scale bar: 10µm. (D) Dorsal view of anterior end of body of *E. paraensei* incubated with 50 µg/mL artesunate for 48h showing swelling [S], blebs [B], low dorsal collar spines [DS] with empty spines pocket [EP]. Scale bar: 20µm. (E)- Dorsal view of posterior region of *E. paraensei* incubated with 50 µg/mL artesunate for 48h showing furrowing [F] and high disruption of tegument showing subtegumental tissue [ST]. Scale bar: 25µm.

Discussion

Food-borne trematode infections, beside other previously neglected tropical infections, have now become priority targets for preventive chemotherapy (mass drug administration) for control or elimination [29]. Despite increased support for treatment and control of food-borne trematode infections in recent decades, praziquantel and tricabendazole are currently the only drugs available against adult worms [4, 5, 6, 10]. Furthermore, drug resistance of adult worms has been highlighted as an increasing threat [30]. All food-borne trematodes have complex life cycles, including numerous definitive hosts and various snail species acting as intermediate hosts, most of which are specific to a particular trematode species [31]. As a consequence, their maintenance under laboratory conditions can be an expensive, time consuming and potentially wasteful practice, or can require infrastructure that hinders the development of research. Moreover, drug testing involves exposing host laboratory animals to experimental infection to determine the effectiveness of treatment, requiring killing the animals at specified time points after treatment [32].

The difficulties mentioned above can be overcome by using three strategies. The first is to have an inexpensive and short-lived experimental life cycle that reproduces parasites that cause food-borne trematode infections. The most recent research indicates that several species of the genus *Echinostoma* satisfy this need [24, 33]. Our previous results showed that *E. paraensei* is a useful model for both biological studies [34, 35, 36] and therapeutic studies of the use of praziquantel, which causes worm burden reduction and damages at the surface and ultrastructural level [7, 9]. The second is to investigate extracts from plants used in traditional medicine that are potential sources of bioactive compounds, including antiparasitic activity [37]. Since the early 1970s, the Chinese herb *Artemisia annua*, the source of artemisinin and its major derivatives (artemether, artesunate and dihydroartemisinin), has been studied for this purpose [38]. Both *in vitro* and *in vivo* studies have revealed the effect of artesunate against *E.*

caproni adults [19]. The third is to carry out *in vitro* assays against juvenile stages as targets to select new active compounds [33].

Due to the increasing interest in natural products [39, 40], we analyzed the effect of artesunate on newly excysted metacercariae. Five major points can be mentioned regarding our results. The first relates to the ideal conditions for excystation, in which a considerable number of juvenile worms are obtained in about two hours [24]. The next step was to have an *in vitro* model in a defined medium that is easy, rapid and cheap to test potential compounds with anthelmintic activity on newly excysted metacercariae. Although the mechanism of action by which artesunate acts against *E. paraensei* has not been elucidated in detail, the possible mechanism and biochemical pathway of artemisinin derivatives has been highlighted for other flukes. The drug can generate oxidative damage to parasite membranes and disruption of the electron transport chain in mitochondria [41]. Artemether inhibits Na^+-K^+-ATPase and Mg^{2+}-ATPase [42], reduces the enzyme activities of phospofructokinase, phosphogycerate kinase, pyruvate kinase and lactate dehydrogenase [43, 44], increases glycogen phosphorylase activities [45] and causes disruption of the cytoskeleton and osmoregulatory system [23].

The second point relates to fact that artesunate has been shown to cause similar response in immature *F. hepatica* [19], *F. gigantica* [26] and *S. mansoni* [46, 47, 48, 49, 50, 51]. Another important result of our study is the marked modification in motility and tegument damage. In this context, it is important to emphasize that these effects are both time and dose-dependent, in which short exposure times and low doses do not seem to cause any change in motility or morphology. When the exposure time and concentration of artesunate increase, the changes in the motor activity, tegument damage and death increase. At low concentrations (1 μg/mL or 10 μg/mL) during longer exposure periods, artesunate causes

decreased movement and tegument change similar to those of worms exposed to 25 µg/mL for 12-24 h or 50 µg/mL for 24 h.

Motility declined substantially in worms exposed to 25 µg/mL for 24 h. The posterior body region was paralyzed, narrowed and somewhat curved ventrally, while the forebody was contracted, suggesting that such changes may be related to a direct effect at the musculature level. This observation is in line with results showing cercariae with complete immobility of the tails but the heads having twitching movements after exposure to mefloquine and primaquine [52].

Light microscopic examination of praziquantel-treated adult *E. paraensei* showed shortened body length with contracted musculature [7]. Interestingly, the ethanol extract of artemisin dislodged the monogenean parasite *Heterobranchus longifilis* from its attachment organs [53]. Given the similarity in response to each compound, it is plausible that praziquantel and artemisin have the same action mechanism.

The third point relates to the excretory tubules filled with enlarged calcareous corpuscles. Even though these mineral concretions are produced by parasitic platyhelminths, the exact mechanisms of action remain elusive [54]. Scanning and transmission electron microscopy of the female reproductive system of *Schistosoma margrebowiei* revealed calcareous corpuscles within mature S4 cells in the vitelline gland [55]. During the developmental process from protoscolex to adult worm in tapeworms, the calcareous corpuscles disappear in the first days of infection. This fact supports the hypothesis that they are sources of either energy or ions for the initial establishment and development in the definitive host [56]. Other authors suggest that calcareous corpuscles are source of calcium carbonate and a play a role in tissue repair [57].

The forth point is that higher concentrations for longer exposure periods to artesunate lead to shrunken and widened bodies and tissue disorganization within undifferentiated internal organs. To further describe the effects of artesunate at the ultrastructural level, we performed scanning electron microscopic analysis. As expected, untreated worms were normal in appearance. Incubation with 100 µg/mL for 24 h promoted mild changes in the anterior region (slight swelling, tegument with disordered spines, blebs and holes). A time-dependent relationship was evident for worms exposed to 50 µg/mL for 48 h. This resulted in severe damage to the anterior region around the oral sucker, with high swelling, including disorganization of the spine-bearing collar (peristomic collar or cephalic collar) and papillae deformity. Our findings are consistent with those in the literature, reporting tegument morphological alterations (eruption, blebs, furrowing, sloughing, swelling, disruption and deformity) provoked by artemisin derivates *in vitro* on *Schistosoma mekongi* [17], *F. hepatica* [19], *F. gigantica* [22, 58] and *E. caproni* [4].

In the present study, the anterior region of *E. paraensei* had a large number of uniciliated and domed papillae, similar to other echinostomes [59]. Adult *E. paraensei* showed a sensory region between the collar spines and sucker lips, whereas three rows of papillae were present in the dorsal collar spines. The acetabular region contained small ciliated and domed papillae distributed randomly [60]. This implies that among species of the family Echinostomatidae (*E. paraensei* and *Echinoparyphium recorvatum*), differences regarding the number and distribution of papillae may be related to the developmental stage [60]. Although the mechanism by which papillae play their role is not known, we suggest that the kind and number of papillae in the worm's body play a pivotal role regarding the orientation on the position of the body, as well as recognize their infection site. Our results suggest that the attachment ability and behavioral pattern may be impaired [61].

The fifth point relates to the killing of parasites as the last step to determine the efficacy of compounds exhibiting anthelmintic activity against flatworms. In this regard, flukes display differences in susceptibility to artesunate and derivates [19]. For instance, the total mortality rate of *F. hepatica* exposed to 100 μg/mL of artesunate was reached after 72 h of incubation [19]. In the current investigation, this concentration promoted total parasite death after 24 h of incubation. When compared with previously published observation periods for adult *E. caproni* specimens [19], we noted that complete worm reduction is also achieved with the same time of incubation. Moreover, no adult *E. paraensei* was recovered from mice 24 h after treatment with 100 mg/kg of praziquantel [7].

In the present study, dead worms were paralyzed and did not show movement during observation for 2 min. In analyzing the phenotypic response to artesunate, the worms were characterized by their dark appearance, atypical body shape, highly vacuolated parenchyma, undifferentiated internal organs and calcareous corpuscles. The integrity and function of the tegument are critical to the survival of flatworms within the vertebrate host. Although staining with vital dye was not performed to test the membrane permeability, we can hypothesize that physiological processes, such as access to nutrients present in the environment, may be impaired. As yet there is no available marker for use in this type of drug-sensitivity assay [8].

The greatest achievements in medicine in the 19th and 20th centuries were possible due to the use of animals as experimental models [62]. However, this practice has come under increasing challenge since the early 1960s, when the 3Rs principle (Replacement, Reduction and Refinement) was proposed for ethical research and testing using animals [63]. Today, it is recommended that research should carried out with alternative techniques that provide the same level of information as that obtained in experiments using animals [64]. Moreover, technological improvements of *in vitro* assays will significantly benefit from the 3Rs principle of animal-based methods. This study was in line with these recommendations because the

observed effects were similar to those described for adult worms that are found within experimental animals. Moreover, they were in agreement with previous studies using newly excysted metacercariae of *E. caproni* [8].

Conclusion

In conclusion, we have for the first time shown *in vitro* anthelmintic activity of artesunate against immature *E. paraensei*, and demonstrated that motility and tegument changes were both time and dose-dependent. An important consideration is that our *in vitro* study reproduced results described for other immature flukes, and even for adult *E. paraensei* specimens treated with praziquantel. Our findings make a significant contribution towards testing to find compounds from natural sources, such as Brazilian native flora. Finally, the better researchers understand the usefulness of juvenile parasites for *in vitro* drug screening, the more they can avoid unnecessary death of experimental animals.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

JGRS designed, performed, analyzed, discussed the experiments and drafted the manuscript. EJLT carried out the scanning electron microscopy analysis, interpretation of data and revised the manuscript. JSG maintained the life-cycle of the parasite and revised the manuscript. RRS helped *in vitro* assay and revised the manuscript. AMJ conceived, designed, helped coordinate the study and revised the manuscript. JRMS conceived, designed and coordinated the project, discussed and revised the results and drafted the manuscript.

Author's information

Souza JGR was supported by a doctorial fellowship from Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil) and Machado-Silva is research

fellow from the Brazilian Council for Scientific and Technological Development (CNPq, Brazil).

Acknowledgement

References

1. Ross F, Hernández P, Porcal W, López GV, Cerecetto H, González M, Basika T, Carmona C, Fló M, Maggioli G, Bonilla M, Gladyshev VN, Boiani M, Salinas G.. Identification of thioredoxin glutathione reductase inhibitors that kill cestode and trematode parasites. PLoS One. 2012;7:e35033.

2. Fürst T, Duthaler U, Sripa B, Utzinger J, Keiser J. Trematode infections: liver and lung flukes. Infect Dis Clin North Am. 2012;26:399-419.

3. Keiser J, Utzinger J. Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother. 2004;5:1711-1726.

4. Keiser J, Shu-Hua X, Tanner M, Utzinger J. Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*. J Antimicrob Chemother. 2006;57:1139-1145.

5. Kim CS, Min DY. Synthesis of praziquantel derivatives and their *in vitro* activity against adult *Clonorchis sinensis*. Arch Pharm Res. 1998;21:744-8.

6. Lovis L, Mak TK, Phongluxa K, Ayé Soukhathammavong P, Vonghachack Y, Keiser J, Vounatsou P, Tanner M, Hatz C, Utzinger J, Odermatt P, Akkhavong K. Efficacy of praziquantel against *Schistosoma mekongi* and *Opisthorchis viverrini*: a randomized, single-blinded dose-comparison trial. PLoS Negl Trop Dis. 2012;6(7):e1726.

7. Ferraz J, Souza J, Costa-Silva M, Torres E, Santana A, Lanfredi R, Maldonado A, Garcia J. Effect of praziquantel on adult *Echinostoma paraensei* worms in experimentally infected mice. Parasitol Res. 2012;111:143-148.

8. Panic G, Ingram K, Keiser J. Development of an in vitro drug sensitivity assay based on newly excysted larvae of *Echinostoma caproni*. Parasit Vectors. 2013;237-245.

9. Gonçalves JP, Oliveira-Menezes A, Maldonado Junior A, Carvalho TM, de Souza W. Evaluation of praziquantel effects on *Echinostoma paraensei* ultrastructure. Vet Parasitol. 2013;194:16-25.

10. Chai JY. Paragonimiasis. Handb Clin Neurol. 2013;114:283-96.

11. Xiao SH, Catto BA, Webster LT. Effects of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. J Infect Dis. 1985;151:1130-7.

12. Keiser J, Morson G. *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Exp Parasitol. 2008;118:228-237.

13. Overend DJ1, Bowen FL. Resistance of *Fasciola hepatica* to triclabendazole. Aust Vet J. 1995;72:275-276.

14. Moll L, Gaasenbeek CP, Vellema P, Borgsteede FH. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The netherlands. Vet Parasitol. 2000;91:153-8.

15. Duthaler U, Smith TA, Keiser J. *In vivo* and *in vitro* sensitivity of *Fasciola hepatica* to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob Agents Chemother. 2010;54:4596-4604.

16. Alvarez-Sanchez MA, Mainar-Jaime RC, Pérez-Garcia J, Rojo-Vasquez FA. Resistance of *Fasciola hepatica* to tricabendazole and albendazole in sheep in Spain. Vet Rec 2006,159:424-425.

17. Jiraungkoorskul W, Sahaphong S, Sobhon P, Riengrojpitak S, Kangwanrangsa N. *Schistosoma mekongi*: the *in vitro* effect of praziquantel and artesunate on the adult fluke. Exp Parasitol. 2006;113:16-23.

18. Keiser J, Shu-Hua X, Jian X, Zhen-San C, Odermatt P, Tesana S, Tanner M, Utzinger J. Effect of artesunate and artemether against *Clonorchis sinensis* and *Opisthorchis viverrini* in rodent models. Int J Antimicrob Agents. 2006;28:370-373.

19. Keiser J, Brun R, Fried B, Utzinger J. Trematocidal activity of praziquantel and artemisinin derivatives: *in vitro* and *in vivo* investigations with adult *Echinostoma caproni*. Antimicrob Agents Chemother. 2006;50:803-805.

20. Keiser J, Utzinger J, Vennerstrom JL, Dong Y, Brennan G, Fairweather I. Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. Trans R Soc Trop Med Hyg. 2007;101:1219-1222.

21. Keiser J, Rinaldi L, Veneziano V, Mezzino L, Tanner M, Utzinger J, Cringoli G. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res. 2008;103:517-522.

22. Krishna S, Bustamante L, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. Trends Pharm Sci. 2008;29:520-527.

23. Tansatit T, Sahaphong S, Riengrojpitak S, Viyanant V, Sobhon P. *Fasciola gigantica*: The in vitro effects of artesunato as compared to tricabendazole on the 3-weeks-old juvenile. Exp Parasitol 2012;131:8-19.

24. Fried B, Peoples RO. Maintenance, cultivation, and excystation of echinostomes: 2000-2007. *In*: The biology of echinostomes from de molecule to the community. Freid B, Toledo R (Eds). 2009;112-124.

25. Souza JG; Garcia J; Neves RH; Machado-Silva JR; Maldonado A. *In vitro* excystation of *Echinostoma paraensei* (Digenea: Echinostomatidae) metacercariae assessed by

light, morphometry and confocal laser scanning microscopy. Exp Parasitol 2013;135:701-707.

26. Lorsuwannarat N, Piedrafita D, Chantree P, Sansri V, Songkoomkrong S, Bantuchai S, Sangpairot K, Kueakhai P, Changklungmoa N, Chaichanasak P, Chansela P, Sobhon P. The *in vitro* anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*. Exp Parasitol. 2014;136:5-13.

27. Garcia JS, Hooper, C Simões RO, Santos MAJ, Maldonado A. Silva JP. Biochemical and histological responses of *Rattus novergicus* (Wistar) infected by *Echinostoma paraensei* (Trematoda: Echinostomatidae). Vet Parasitol. 2011;178(1-2):86-92.

28. Lopes Torres, EJ, De Souza W, Miranda K. Comparative analysis of *Trichuris muris* surface using conventional, low vacum, environmental and field emission scanning electron microscopy. Vet. Parasitol. 2013;196, 409-416.

29. Hotez PJ. "Blue marble health"--neglected tropical disease control and elimination in a shifting health policy landscape. PLoS Negl Trop Dis. 2013;7(11):e2570.

30. Andrews KT, Fisher G, Skinner-Adams TS. Drug repurposing and human parasitic protozoan diseases. Int J Parasitol Drugs Drug Resist. 2014;4:95-111.

31. Keiser J, Utzinger J. Food-borne trematodiases. Clin Microbiol. Rev. 2009;22:466-483.

32. Krautz-Peterson G, Ndegwa D,Vasquez K, Korideck H, Zhang J, Peterson JD, Skelly PJ. Imaging schistosomes *in vivo*. The FASEB J. 2009;23:2674-2677.

33. Keiser J. *In vitro* and *in vivo* trematde models for chemotherapeutic studies. Parasitology. 2010;589-603.

34. Maldonado A Jr, Zeitone BK, Amado LA, Rosa IF, Machado-Silva JR, Lanfredi RM. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. J Helminthol. 2005;79:345-351.

35. Souza JGR, Garcia JS, Manso PPA, Neves RH, Maldonado A, Machado-Silva JR. Development of the reproductive system of the *Echinostoma paraensei* in *Mesocricetus auratus* analyzed by light and confocal scanning laser microscopy. Exp Parasitol 2011;128:341-346.

36. Garcia JS, Pinheiro J, Hooper CS, Simões RO, Ferraz JS, Maldonado A. Haematological alterations in *Rattus norvegicus* (Wistar) experimentally infected with *Echinostoma paraensei* (Trematoda: Echinostomatidae). Exp Parasitol. 2012;131:300-3.

37. Liu YX, Wu W, Liang Y J, Jie ZL, Huang YX. New uses for Old Drugs: The tale of artemisinin derivatives in the elimination of schistosomiasis japonica in China. Molecules. 2014;19:15058-15074.

38. Ndjonka D, Rapado LN, Silber AM, Wrenger C. Natural products as a source for treating neglected parasitic diseases. Int. J.Mol. Sci. 2013;14:3395-3439.

39. Cunha NL, Uchoa CJM, Silva L, Souza HC, Peixoto JA, Silva CP, Magalhaes LG, Gimenez VMM, Groppo M, Rodrigues V, Silva-Filho AA, Silva MLA, Cunha WR, Pauletti PM, Januario AH. *In vitro* schistosomicidal activity of some Brazilian Cerrado species and their isolated compounds. Evidence-based complementary and alternative medicine. 2012;1-8.

40. Koné WM, Vargas M, Keiser J. Anthelmintic activity of medicinal plants used in Côte d'Ivoire for treating parasitic diseases. Parasitol Res. 2012;110:2351–2362

41. O'Neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin – The date continues. Moleculate 2010;15:1705-1721.

42. Xiao SH, Booth M, Tanner M. The prophylactic effects od artemether against *Schistosoma japonicum* infection. Parasitol Today 2000;16:122-126.

43. Xiao SH, You JQ, Guo HF, Jiao PY, Mei JY, Yao MY, Feng Z. Effect of artemether on glyceraldehydes-3-phosphate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase of *Schistosomo japonicum* harbored in mice. Acta Pharmacol. Sin. 1998;19:279-81.

44. Xiao SH, You JQ, Guo HF, Jiao PY, Mei JY, Yao MY, Feng Z. Effect of artemether on hexokinase glucose phosphate isomerase and phosphofructokinase of *Schistosoma japonicum* harbored in mice. Chin. J. Parasitol. Para. Dis. 1998;16:25-28.

45. Xiao SH, You JQ, Guo HF, Mei JY, Jiao PY,Yao MY, Zhuang ZN, Feng Z. Effect of artemether on phosphorylase, lactate dehydrogenase, adenosine triphosphatase, and glucosephosphate dehydrogenase of *Schistosoma japonicum* hoarnored in mice. Acta Pharmacol Sin 1999;20:750-754.

46. Xiao S, Catto BA. *In vitro* and *in vivo* studies of the effect of artemether on *Schistosoma mansoni*. Antimicrob Agents and Chemother. 1989;1557-1562.

47. Xiao SH, Chollet J, Weiss NA, Bergquist RN, Tanner M. Preventive effect of artemether in experimental animals infected with *Schistosoma mansoni*. Parasitol Int 2000;49:19-24.

48. Utzinger J, Xiao SH, Tanner M, Keiser J. Artemisinins for schistosomisasis and beyond. Curr Opin Investig Drugs. 2007;105-116.

49. Shuhua X, Binggui S, Utzinger J, Chollet J, Tanner M. Transmission electron microscopic observations on ultrastructural damage in juvenile *Schistosoma mansoni* caused by artemether. Acta Trop. 2002;81:53–61.

50. Liu R, Dong HF, Jiang MS. Artemisinin: the gifts from traditional Chinese medicine not only for malaria control but also for schistosomiasis control. Parasitol Res 2012;110:2071–2074.

51. Liu YX, Wu W, Liang Y J, Jie ZL, Huang YX. New uses for Old Drugs: The tale of artemisinin derivatives in the elimination of schistosomiasis japonica in China. Molecules. 2014;19:15058-15074.

52. Holtfreter MC, Loebermann M, Klammt S, Sombetzki M, Bodammer P, Riebold D, Kinzellbach R, Reisinger EC. *Schistosoma mansoni*: schistosomicidal effect of mefloquine and primaquine *in vitro*. Exp Parasitol. 2011;127:270-276.

53. Ekanem AP, Brisibe EA. Effects of ethanol extract of *Artemisia annua L.* against monogenean parasites of *Heterobranchus longifilis.* Parasitol Res. 2010;106:1135–1139.

54. Chalar C, Salomé M, Senorale-Pose M, Marína M, Williams CT, Dauphind Y. A high resolution analysis of the structure and chemical composition of the calcareous corpuscles from *Mesocestoides corti*. Micron. 2013;44:185–192.

55. Awad AH, Probert AJ. Scanning and transmission electron microscopy of the female reproductive system of *Schistosoma margrebowiei* Le Roux, 1933. J Helminthol. 1990;64:181-92.

56. Rodrigues JJ, Ferreira HB, Farias SE, Zaha A. A protein with a novel calcium-binding domain associated with calcareous corpuscles in *Echinococcus granulosus*. Biochem Biophys Res Commun. 1997;237:451-6.

57. Vargas-Parada L, Merchant MT, Willms K, Laclette JP. Formation of calcareous corpuscles in the lumen of excretory canals of *Taenia solium cysticerci*. Parasitol Res. 1999;85:88-92.

58. Diab TM, Mansour HH, Mahmoud SS. *Fasciola gigantica*: Parasitological and scanning electron microscopy study of the *in vitro* effects of ivermectin and/or artemether. Exp Parasitol. 2010;124:279-84.

59. Ursone RL, Fried B. Light and scanning electron microscopy of *Echinostoma caproni* (Trematoda) during maturation in ICR mice. Parasitol Res. 1995;81:45-51.

60. Maldonado A, Loker ES, Morgan JAT, Rey L, Lanfredi LM. Description of the adult worm of a Brazilian isolate of *Echinostoma parensei* (Platyhelminthes: Digenea) from its natural vertebrate host *Nectomys squamipes* by light and scanning electron microscopy. Parasitol Res 2001;87:840-848.

61. Šebelová S, Sterwart MT, Mousley A, Fried B, Marks JN, Halton DW. The musculature and associated innervation of adult and intramolluscan stages of *Echinostoma caproni* (Trematoda) visualized by confocal microscopy. Parasitology Research. 2004;93:196-206.

62. Flecknell P. Replacement, reduction and refinement. ALTEX. 2002;19:73-8.

63. Russell WMS, Burch RL. The Principles 0f Humane Experimental Technique. London: Methuen and Co. Ltd. 1959.

64. Eskes C, Sá-Rocha Vde M, Nunes J, Presgrave O, de Carvalho D, Masson P, Rivera E, Coecke S, Kreysa J, Hartung T. Proposal for a Brazilian centre on alternative test methods. ALTEX. 2009;26:303-6.

5 **DISCUSSÃO**

Desde que aconteceu a descoberta do *N. squamipes* como hospedeiro natural do *E. paraensei* (Maldonado et al., 2001), publicações posteriores (Maldonado et al., 2003; Pinheiro et al., 2004a,b; 2005; 2009) evidenciaram a viabilidade de manutenção do ciclo biológico em condições de laboratório. Semelhante a outras espécies do gênero *Echinostoma* (Toledo et al., 2004a,b), o hamster (*Mesocricetus auratus*) tem se mostrado o hospedeiro mais adequado para a manutenção do trematódeo em laboratório. Esta tese teve como principal objetivo relatar conhecimentos mais específicos, a respeito do desenvolvimento e manutenção do trematódeo *E. paraensei* nos modelos *in vivo* e *in vitro*. Para tal, destaco diferentes momentos: Dados da literatura mostram que o hamster e o rato Wistar apresentam graus diferentes de suscetibilidade ao *E. paraensei*. Tal resultado é baseado nos achados sobre as características dos vermes adultos isolados de cada hospedeiro. Consequentemente, outros pontos devem ser abordados: o momento da infecção que essa diferença possa ser evidenciada; o desenvolvimento morfométrico e morfológico analisados por microscopia de luz e confocal são critérios para caracterizar essa diferença e quais as estruturas que são afetadas.

Estudos prévios têm demonstrado que espécies do gênero *Echinostoma* são dependentes de fatores relacionados ao hospedeiro. Por exemplo, sabe-se que existem diferenças em relação ao estabelecimento, sobrevivência, oviposição, taxa de recuperação, variáveis morfométricas, parâmetros imunológicos e expulsão de *E. caproni* e *E. trivolvis*, em função do tipo de hospedeiro: hamsters, camundongos ou rato Wistar (Hansen et al., 1991, Weinstein & Fried, 1991, Kruse et al., 1992, Toledo el al., 2004a, b; 2006).

Em concordância com os dados da literatura (Toledo el al., 2004a, b), nesse estudo foi verificado que *E.paraensei* apresenta desenvolvimento morfológico e morfométrico variado, em função da espécie de hospedeiro. Diferente do até então conhecimento da literatura, foi visto que os parâmetros morfométricos analisados de helmintos jovens (3 dias) à ovígeros (21 dias), sofreram alterações caracterizadas como sendo hospedeiro definitivo específicas. Os helmintos de hamsters mostraram um maior desenvolvimento estatisticamente significativo do que aqueles isolados de rato Wistar. Além disso, alterações morfológicas nos helmintos jovens (7 dias) como extrusão do parênquima e outras pontuais no trato reprodutor, como ausência de glândulas vitelínicas e atrofia dos ovários, foram observadas nos poucos helmintos que se mantiveram em rato. O desenvolvimento de alterações no sistema

reprodutor, parcialmente, reproduziram observações feitas também no trematódeo *S. mansoni*, em comparação com o camundongo albino que é um hospedeiro de maior compatibilidade (Silva-Leitão et al., 2009). Esses dados sugerem que a maior ou menor permissividade de um hospedeiro se manifesta desde o início da infecção. Entretanto, não se conhece os fatores determinantes dessas diferenças na relação parasito-hospedeiro.

Para a maior parte das espécies de *Echinostoma* que infectam roedores, o crescimento foi estudado por meio de medidas lineares. Estas indicaram uma fase inicial lenta de crescimento nos primeiros três dias de infecção seguida de um rápido crescimento após esse período (Mohandas & Nadakal, 1978). O mesmo processo ocorreu para *E. paraensei* porém, o maior crescimento se deu a partir do sétimo dia de infecção. Além disso, o desenvolvimento dos órgãos do sistema reprodutor também foi mais tardio tanto em hamster quanto em rato quando comparado com outras espécies do gênero (Mohandas and Nadakal., 1978; Seo et al., 1985; Franco et al., 1986; Fried et al, 1988; Toledo el al., 2004a, b).

A diferença de compatibilidade entre o parasito e seus hospedeiros também foi evidenciada na taxa de recuperação de helmintos ao longo do tempo. Em hamsters, a recuperação foi maior e estável, enquanto que em rato houve uma diminuição em função da idade de infecção. Há várias indicações de que essa diminuição de recuperação esteja relacionada com a expulsão dos vermes do seu microambiente (Weinstein & Fried, 1991). Os dados sugerem que modificações na estrutura do intestino delgado, como a erosão das vilosidades intestinais acrescida de notável aumento de células caliciformes (Toledo et al., 2006) ou diferenças na resposta imune (Trelis et al., 2011; Cortes et al., 2014).

Os dados analisados nesse trabalho estão de acordo com aqueles obtidos em estudos anteriores que avaliaram o grau de compatibilidade desses dois hospedeiros a infecções por outros trematódeos (Toledo el al., 2004a; Silva-Leitão et al., 2009). Nós concluímos que o rato se constitui em ambiente para o desenvolvimento morfológico e morfométrico de *E. paraensei*, caracterizando o seu menor nível de compatibilidade, já é expresso desde as formas juvenis. Além disso, o presente estudo contribui para uma descrição fenotípica mais completa contemplando relações de crescimento entre estruturas (alometria).

O segundo momento foi acompanhar as características do cultivo *in vitro* do *E. paraensei*. Para tal, inicialmente, foram estudadas as fases do desencistamento das metacercárias de *E. paraensei,*descrevendo a morfologia das larvas recém desencistadas, aspectos até então não conhecidos para esta espécie. Até o presente momento, dentre os estudos sobre desencistamento de espécies de *Echinostoma*, apenas uma tentativa foi feita

sem sucesso para desencistar *E. paraensei* (Fried & Reddy, 1997). Além disso, já se sabia sobre a morfologia das diferentes formas evolutivas do ciclo biológico desse parasito com exceção de larvas desencistadas. A maior expectativa foi criada em tentar obter material biológico vivo, que pudesse ter semelhança com maior grau possível aos helmintos adultos sem haver a necessidade de sacrifício do hospedeiro definitivo em um curto espaço de tempo.

Para que pudéssemos confirmar se as taxas de desencistamento obtidas em estudos anteriores eram específicas, nós verificamos em diferentes tempos de exposição, a dependência da metacercária de *E. paraensei* à diferentes concentrações e presença/ausência dos principais componentes do meio de desencistamento: a tripsina e os sais de bile. Nessa primeira etapa concluímos que para esta espécie, as taxas de desencistamento e os estágios pelas quais a metacercária passa até ficar livre no meio (encistada, ativada, rompida e desencistada) eram similares àquelas relatadas para as demais espécies do gênero (*E. caproni*, *E. lie*, *E. revolutum* e *E. trivolvis*) (Fried & Butler, 1978; Fried & Emili, 1988; Smoluk & Fried, 1994; Ursone & Fried, 1995; Fried & Reddy, 1997; Fried & Peoples, 2007; Saxton et al., 2008), não havendo diferenças em relação às concentrações de tripsina e sais de bile e necessidade de um tratamento ácido. Apesar de alcançar as mesmas taxas de desencistamento ao final de 2 h, *E. paraensei* necessita de mais tempo do que as espécies acima citadas para que o fenômeno ocorra. Por esse estudo, verificamos que os sais de bile são responsáveis pela fase de ativação da metacercária dentro do cisto. Observamos que as metacercárias recém desencistadas eram morfologicamente semelhantes aos helmintos adultos com exceção do sistema reprodutor e da presença de concreções excretoras nos túbulos excretores. *E. paraensei* difere das demais espécies do gênero também pela quantidade de concreções excretoras presentes no túbulo excretor logo após o desencistamento. As concreções desaparecem ao passar do tempo. Este fato ainda não foi elucidado para as espécies de *Echinostoma*, porém, sugerimos que sejam utilizadas como fontes de íons durante as intensas atividades musculares características nessa fase do helminto ou ainda, sugerimos que tais concreções possam estar envolvidas no processo de morfogênese, principalmente devido a localização. Nesse momento, também foi confirmado a presença de uma região digerida para a saída da metacercária do cisto, como elucidado para outros trematódeos (Irwin et al., 1990). Finalmente, adicionamos novos dados morfométricos de larvas recém desencistadas incluindo a relação entre diferentes estruturas.

O terceiro momento foi aplicar o conhecimento obtido com as etapas de cultivo e o desencistamento das metacercárias, como fonte de material biológico. O estudo do

desencistamento propiciou amplo conhecimento a respeito da obtenção de material biológico fresco em número considerável, num curto espaço de tempo (2 h). Esse fato foi um dos motivadores para que fosse testada *in vitro*, a ação do artesunato, cuja atividade antiparasitária já tem sido comprovada em vermes adultos (Xiao et al., 1996, 1997, 1998a; Jiraungkoorskul et al., 2006; Keiser et al., 2006; Keoser e Morson, 2008; Tansatit et al., 2012; Diab et al., 2010). Cabe destacar que o artesunato é um produto amplamente utilizado na medicina chinesa como antimalárico (Klayman, 1985; De Vries & Dien, 1996; Ziffer et al., 1997; Van Agtmael et al., 1999; VRon et al., 1999; Dhingra et al., 2000; Price et al., 2000). Como principais impactos desse tipo de estudo podem ser citados: os aspectos éticos pelo enquadramento do projeto de pesquisa entre aqueles que atendem a atual política dos 3Rs cujo tema é refinar, reciclar e substituir (Refine, Recycle e Replace) (Panic et al., 2013). Além do melhor refinamento do experimento, poderá haver a implantação de metodologias alternativas, com maior racionalidade para os testes de drogas, condicionando o sacrifício dos animais de experimentação. Além disso, associamos do modelo à necessidade de descoberta de novas drogas ou com pouco conhecimento, devido à uma crescente preocupação quanto a existência de resistência ou diferença no nível de tolerabilidade as atuais drogas de escolha n tratamento de infecções transmitidas por alimentos: o praziquantel e o triclabendazol (Keiser & Utzinger, 2004). Foi então, pensado num modelo *in vitro*, utilizando as larvas recém desencistadas de *E. paraensei* para testes da ação de compostos, substâncias ou fármacos que pudesse ter o seu potencial anti-helmíntico testado. O extrato sintético do artesunato foi avaliado em cinco concentrações e em cinco tempos diferentes de exposição. A análise da ação *in vitro* deste fármaco foi feita por meio de análise microscópica quantitativa e qualitativa dos helmintos por microscopia de luz, enquanto possíveis alterações na superfície do tegumento foram estudadas por microscopia eletrônica de varredura.

No ensaio atual, as larvas recém desencistadas foram sensíveis às maiores concentrações de artesunato (100 e 50 µg/mL) em menores tempos testados (24 e 48 h, respectivamente). Alterações na atividade motora, mortalidade e danos no tegumento como vesículas, inchaços, dobras, orifícios, perda de espinhos do colar e tegumento foram documentados. Entretanto, os mecanismos de ação de derivados da artemisinina não são bem elucidados na literatura. Sabe-se que um dos derivados, o arteméther provoca alterações bioquímicas em espécies de *Schistosoma* inibindo a atividade de enzimas importantes da via glicolítica e na bomba de sódio-potássio e de magnésio deste parasito (Xiao et al., 1998a, b; 1999; 2000). As alterações morfológicas causadas pelo artesunato no presente estudo também

foram observadas em espécies jovens e adultas de *F. hepatica* (Keiser & Morson 2008; Duthaler et al., 2010), *F. gigantica* (Diab et al., 2010; Tansatit et al., 2012), *S. mekongi* (Jiraungkoorskul et al., 2006), *S. mansoni* (Mtsiu et al., 2009) e *S. japonicum* (Xiao et al., 2003), submetidas *in vitro* a presença de derivados da artemisinina. A ação do artesunato na paralisia do helminto nos faz propor que talvez este fármaco tenha o mecanismo de ação semelhante a do praziquantel.

Contudo, evidenciamos que o presente modelo *in vitro*, que utiliza larvas de metacercárias recém desencistadas, é sensível e vantajoso para pesquisas que objetivam testar fármacos com potencial anti-helmíntico, principalmente aqueles de ação em formas jovens, evitando o uso de animais de experimentação em fases ainda que preliminares de teste. Este modelo supera algumas dificuldades encontradas em estudos *in vivo*, tais como, substitui o uso de um trematódeo com o ciclo de vida mais longo (tempo), complexo (diferentes hospedeiros definitivos e intermediários) e de manutenção em laboratórios mais dispendiosa ou ainda impossível de manter em condições de laboratório por requerer complexa infraestrutura. Por fim, destacamos que não há estudos anteriores sobre a ação de fármacos sobre formas jovens *E. paraensei*, adicionamos novos dados morfológicos como a indicação de papilas e ainda não destacados na literatura.

REFERÊNCIAS

Abdel-Aziz SS, el-Badawy NM. Experimental trials of an artemisinin derivative in treatment of *Schistosoma mansoni* infected mice. J Egypt Soc Parasitol. 2000; 30: 295–303.

Alvarez-Sanchez MA, Mainar-Jaime RC, Pérez-Garcia J, Rojo-Vasquez FA. Resistance of *Fasciola hepatica* to tricabendazole and albendazole in sheep in Spain. Vet Rec. 2006;159, 424-425.

Anazawa K. On a human case of *Echinostoma revolutum* and its infection route. Taiwan Iggakai Zasshi. 1929; 288, 221-241.

Andrews KT, Fisher G, Skinner-Adams TS. Drug repurposing and human parasitic protozoan diseases. Int J Parasitol Drugs Drug Resist. 2014; 4, 95-111.

Araújo N, Kohn A, Katz N. Activity of the artemether in experimental *schistosomiasis mansoni*. Mem Inst Oswaldo Cruz . 1991; 86 Suppl 2:185-188.

Awad AH, Probert AJ. Scanning and transmission electron microscopy of the female reproductive system of *Schistosoma margrebowiei* Le Roux, 1933. J Helminthol. 1990;64, 181-92.

Balint GA. Artemisinin and its derivatives. An important new class of antimalarial agents. Pharmacol. Therapeut. 2001; 90, p. 261-265.

Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J. Light and electron microscopy studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum* and *Fasciola hepatica* (Trematoda) *in vitro*. Z Parasitenkd. 1980; 63, 113-128.

Beckmann S, Grevelding CG. Imatinib has a fatal impact on morphology, pairing stability and survival of adult *Schistosoma mansoni in vitro*. Int J Parasitol . 2010; 40: 521–526.

Bock D. Hatching mechanism of the metacercriae of *Plagiorchis* species (Trematoda: Plagiorchiidae). J Helminthol. 1989; 63, 153-171.

Bonfim TCS, Maldonado A, Tunholi VM, Tunhol-Alves VM, Faro MJ, Mota EM, Barros TCS, Pinheiro S, Garcia SJ. Biochemical and histhological alterations in *Biomphalariaglabrata* due to co-infection *Angiostrongylus cantonensis* and *Echinostoma paraensei*. J Invertebr Pathol . 2014; 115, 80-85.

Brennan GP, Fairweather I, Trudgett A, Hoey E, McCoy, McConville M, Meaney M, Robinson M, McFerran N, Ryan L, Lanusse C, Mottier L, Alvarez L, Solana H, Virkel G, Brophy PM. Understanding tricabendazole resistance. Exp Mol Pathol. 82,104-109. Chai JY. 2013. Paragonimiasis. Handb Clin Neurol . 2007; 114, 283-96.

Chalar C, Salomé M, Señorale-Pose M, Marin M, Williams C.T., Dauphin Y. A high resolution analysis of the structure and chemical composition of the calcareous corpuscles from Mesocestoides corti. Micron. 2013; 44:185-92.

Chen, DJ, Fu LF, Shao PP, Wu FZ, Fan CZ, Shu H, Ren CS, Sheng XL. Studies on antischistosomal activity of qinghaosu in experimental therapy. Zhong Hui Yi Xue Zha Zhi. 1980; 80, 422 –8.

Cortés A, Muñoz-Antoli C, Sotillo J, Fried B, Esteban JG, Toledo R. *Echinostoma caproni* (Trematoda): differential *in vivo* mucin expression and glycosylation in high- and low-compatible hosts. Parasite Immunol. 2015; 37, 32-42

Chung YB, Kong Y, Joo IL, Cho SY, Kang SY. Excystment of *Paragonimus westermani* by endogenous cysteine protease. J Parasitol. 1995; 81:137-142.

Cunha NL, Uchoa CJM, Silva L, Souza HC, Peixoto JA, Silva CP, Magalhaes LG, Gimenez VMM, Groppo M, Rodrigues V, Silva-Filho AA, Silva MLA, Cunha WR, Pauletti PM, Januario AH. *In vitro* schistosomicidal activity of some Brazilian Cerrado species and their isolated compounds. Evidence-based complementary and alternative medicine. 2012; 1-8.

Desses S.S. Calcium accumulation in larval *Echinoccus multilocularis*. Can J Zool. 1963; 41: 1055-1059.

Dhingra V, Vishweshwar Rao K, Lakshmi Narasu, M. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci. 2000; 66: 279–300.

Diab TM, Mansour HH, Mahmoud SS. *Fasciola gigantica*: Parasitological and scanning electron microscopy study of the *in vitro* effects of ivermectin and/or artemether. Exp Parasitol. 2010; 124, 279-84.

Doenhoff MJ, Kusel JR, Coles GC, Cioli D. Resistance of *Schistosoma mansoni* to praziquantel: is there a problem? Trans R Soc Trop Med Hyg. 2002; 96, 465-469.

Duthaler U, Smith TA, Keiser J. *In vivo* and *in vitro* sensitivity of *Fasciola hepatica* to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob Agents Chemother. 2010; 54:4596-4604.

de Vries, PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. Drugs. 1996; 52: 818 –36.

Eckstein-Ludwig, U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PM, Ward SA, Khrisna S. Artemisinins target the SERCA of *Plasmodium falciparum*. Nature. 2003; 424, 957–961.

Ekanem AP, Brisibe EA. Effects of ethanol extract of *Artemisia annua L.* against monogenean parasites of *Heterobranchus longifilis*. Parasitol Res. 2010; 106, 1135–1139.

Ellery, AW. Guidelines for specification of animals and husbandry methods when reporting the results of animal experiments. Working Committee for the Biological Characterization of Laboratory Animals/GV-SOLAS. Laboratory Animals. 1985; 19, 106–108.

Eskes C, Sá-Rocha Vde M, Nunes J, Presgrave O, de Carvalho D, Masson P, Rivera E, Coecke S, Kreysa J, Hartung T. Proposal for a Brazilian centre on alternative test methods. ALTEX. 2009; 26, 303-6.

Ferraz J, Souza J, Costa-Silva M, Torres E, Santana A, Lanfredi R, Maldonado A, Garcia J. Effect of praziquantel on adult *Echinostoma paraensei* worms in experimentally infected mice. Parasitol Res. 2012; 11, 143-148.

Flecknell P. Replacement, reduction and refinement. ALTEX . 2002;19, 73-8.

Franco J, Huffman JE, Fried B. Infectivity, growth and development of *Echinostoma revolutum* (Digenea: Echinostomatidae) in the golden hamster, *Mesocricetus auratus*. J. Parasitol. 1986; 72, 142-147.

Frédérich M, Dogné JM, Angenot L, De Mol P. New Trends in Anti-Malarial Agents. Curr. Med. Chem. 2002; 9, 1435-1456.

Fried, B. Excystation of metacercariae of *Posthodiplostomum minimum minimum* Hoffman, 1958, and their development in the chick and on the chorioallantois. J. Parasitol .1970; 56, 944-946.

Fried, B., Roth, R.M., 1974. *In vitro* excystment of the metacercariae of *Parorchis acanthus*. J. Parasitol 60, 465.

Fried B, Stromberg BE. *In vitro* excystment of metacercariae of *Fascioloides magma* (Trematoda). J Parasitol. 1985; 71, 263-264.

Fried B, Donovick RA, Emili S. Infectivity, growth and development of *Echinostoma lie* (Trematoda) in the chick. Parasitology. 1988; 82:413-414.

Fried B. Metacercarial excystament of trematode. Adv parasitol. 1994; 33:91-144.

Fried B, Reddy A. Comparative studies on excystation and morphological features of the metacercariae of *Echinostoma paraensei* and *E. caproni*. Int J Parasitol. 1997; 27, 899-901.

Fried B. Maintenance, cultivation and excystation of echinostomes. In Echinostomes as experimental models for biological research, eds. Fried B and Graczyk TK. 2000; 99-118.

Fried B, Schneck JL, Ponder EL. Effect of tonicity, digestive enzymes and bile salts, and nutrient media on the survival of excysted metacercariae of *Echinostoma caproni*. Parasitol Res. 2004; 93, 527-530.

Fried B, Graczyk TK. Recent advances in the biology of *Echinostoma* species in the "revolutum" group. Adv Parasitol. 2004; 29:163-210.

Fried B, Graczyck TK, Tamang L. Food-borne intestinal trematodiases in humem. Parasitol Res. 2004; 93: 159-170.

Fried B, Graczyck TK. Recent advances in the biology of *Echinostoma* species in the revolutum group. Adv Parasito. 2004; l58, 139-195.

Fried B, Johnson PTJ. *In vitro* excystment of metacercariae of *Ribeiroia ondratae*. Parasitol Res. 2005;95, 293-295.

Fried B, Reddy A. Postmetacercarial changes in *Echinostoma caproni* maintained in a defined medium plus calf serum. Korean J. Parasitol. 2007; 38, 173– 175.

Fried B, Peoples RC. Viability of the encysted metacercariae of Echinostoma caproni judged by light microscopy versus chemical excystation. J. Parasitol. 2007; 93, 947.

Fried B, Toledo R. The biology of Echinostomes – from the molecule to the community. Springer. New York, USA. 2009;333p.

Fried B, Peoples RO. Maintenance, cultivation, and excystation of echinostomes: 2000-2007. In: The biology of echinostomes from de molecule to the community. Freid B, Toledo R (Eds). 2009; 112-124.

Fujino T, Fried B, Hosier DW. The expulsion of *Echinostoma trivolvis* (Trematoda) from ICR mice: extension/retraction mechanisms and ultrastructure of the collar spines. Parasitol Res.1994; 80(7):581-7.

Fujino T, Nakano T, Washioka H, Tonosaki A, Ichikawa H, Fried B. Comparative ultrastructure of eggs in *Echinostoma paraensei, E. caproni, and E. trivolvis* (Trematoda: Echinostomatidae). Parasitol Res. 2000; 86, 427-30.

Fürst T, Duthaler U, Sripa B, Utzinger J, Keiser J. Trematode infections: liver and lung flukes. Infect Dis Clin North Am. 2012; 26, 399-419.

Garcia JS, Maldonado A, Bidau JC, Correa LR, Lanfredi RM, Coelho PMZ. Effect of early infection with *Echinostoma paraensei* on interaction *Schistosoma mansoni* with *Biomphalaria glabrata* and *Biomphalaria tenagophila*. Mem Inst Oswaldo Cruz. 2010; 105, 499-503.

Garcia JS, Hooper, C Simões RO, Santos MAJ, Maldonado A. Silva JP. Biochemical and histological responses of *Rattus novergicus* (Wistar) infected by *Echinostoma paraensei* (Trematoda: Echinostomatidae). Vet Parasitol. 2011; 178, 86-92.

Garcia JS, Pinheiro J, Hooper CS, Simões RO, Ferraz JS, Maldonado A. Haematological alterations in *Rattus norvegicus* (Wistar) experimentally infected with *Echinostoma paraensei* (Trematoda: Echinostomatidae). Exp Parasitol. 2012; 131, 300-3.

Gonçalves JP, Oliveira-Menezes A, Maldonado Junior A, Carvalho TM, de Souza W. Evaluation of Praziquantel effects on *Echinostoma paraensei* ultrastructure.Vet Parasitol . 2013;194, 16-25.

Gavidia CM, Gonzalez AE, Barron EA, Ninaquispe B, Llamosas M, Verastegui MR, Robinson C, Gilman RH. Evaluation of oxfendazole, praziquantel and albendazole against cystic echinococcosis: a randomized clinical trial in naturally infected sheep. PLoS Negl Trop Dis. 2010; 4(2): e616.

Graczyk TK, Shiff CJ. Excystment *in vitro* of *Notocotylus attenuatus* (Trematoda: Notocotylidae) metacercariae. J Parasitol. 1993; 79, 92-97.

Graczyk TK, Fried B. Echinostomiasis: a common but forgotten food-borne disease. Am J Trop Med Hyg. 1998; 58, 501-504.

Hansen K, Nielsen JW, Hindsbo O, Christensen NO. *Echinostoma caproni* in rats: worm population dynamics and host blood eosinophilia during primary 6, 25 and 50 metacercarial infections, and resistance to secondary and superimposed infections. Parasitol Res. 1991; 77, 686-690.

Haynes R K, Chan WC, Wong HN, Li KY, Wu WK, Fan KM, Sung HH, Williams ID, Prosperi D, Melato S, Coghi P, Monti D. Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and antimalarial mechanism of action. Chem Med Chem. 2010; 5, 1282–1299.

Hartwig, C. L. et al. Accumulation of artemisinin trioxane derivatives within neutral lipids of *Plasmodium falciparum* malaria parasites is endoperoxide-dependent. Biochem. Pharmacol. 2009; 77, 322–336.

Haselwood GAD. The biologica importance of bile salts. Frontiers in Biology. North Holland, Amsterdan. 1978.

Hien TT, White NJ. Qinghaosu. Lancet . 1993; 341, 603 –608.

Holtfreter MC, Loebermann M, Klammt S, Sombetzki M, Bodammer P, Riebold D, Kinzellbach R, Reisinger EC. *Schistosoma mansoni*: schistosomicidal effect of mefloquine and primaquine in vitro. Exp Parasitol. 2011; 127, 270-276.

Hotez PJ. "Blue marble health"--neglected tropical disease control and elimination in a shifting health policy landscape. PLoS Negl Trop Dis. 2013; 7(11):e2570.

Hotez P. Huffman JE, Stromberg B, Fried B. Proteases from the intramolluscan and vertebrate stages of *Echinostoma trivolvis* and *Echinostoma caproni*. Inter J for Parasitol. 1993; 23.

Huffman JE, Fried B. *Echinostoma* and echinostomiasis. Adv Parasitol. 1990; 29: 215-269.

Humphries JE, Fried B. Histological and histochemical studies on the paraesophageal glands in cercariae and metacercariae of *Echinostoma revolutum* and *E trivolvis*. J Helminthol . 1996; 70, 299-301.

Irwin SWB, McKerr G, Judge BC, Moran I. Studies on metacercarial excystment in *Himasthla leptosoma* (Trematoda: Echinostomatidae) and newly emerged metacercariae. Int J Parasito. 1984; l4, 415–421.

Irwin SWB, Fried B. Scanning an transmission electron microscopic observation on metacercariae of *Echinostoma trivolvis* and *Echinostoma caproni* during in vitro excystation. J Helminthol Society Wash . 1990; 57, 79-83.

Irwin SWB. O'Kane MB, Fried B. Physicochemical conditions necessary for the *in vitro* excystament of *Zygocotyle lunata* (Trematoda: Paramphistomatidae) Parasitol Res. 1993; 79, 416-420.

Jiraungkoorskul W, Sahaphong S, Sobhon P, Riengrojpitak S, Kangwanrangsan N. *Schistosoma mekongi*: The *in vitro* effect of praziquantel and artesunate on the adult fluke. Exp. Parasitol. 2006; 113:16-23.

Johnston BR, Halton DV. *In vitro* excystation of Bucephaloides gracilescens metacercariae (Trematoda: Bucephalidae). Zeitschrift für Parasitenkunde. 1981; 65, 71-78.

Khalifa RM, Mazen NA, Marawan AM, Thabit HT. Histochemical and ultrastructural studies on the calcareous corpuscles and eggs of *Taenia taeniaeformis* and Dipylidium caninum. J Egypt Soc Parasitol. 2011; 41, 513-28.

Kanev I, Fried B, Radev V. Collar spine models in the genus *Echinostoma* (Trematoda: Echinostomatidae). Parasitol Res. 2009; 105, 921-927.

Keiser J, Utzinger J. Chemotherapy for major food-borne trematodes: a review. Exp. Opin Pharmacother. 2004 ;5, 1711-1726.

Keiser J, Engels D, Buscher G, Utzinger J. Tricabendazole for the treatment of fasciolosis and paragonimiasis. Exp Opin Invetig drugs. 2005; 14, 1513-1526.

Keiser J, Shu-Hua X, Tanner M, Utzinger J. Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*. J Antimicrob Chemother. 2006a; 57:1139-1145.

Keiser J, Shu-Hua X, Jian X, Zhen-San C, Odermatt P, Tesana S, Tanner M, Utzinger J.. Effect of artesunate and artemether against *Clonorchis sinensis* and *Opisthorchis viverrini* in rodent models. Int J Antimicrob Agents. 2006b; 28:370-373.

Keiser J, Brun R, Fried B, Utzinger J. Trematocidal activity of praziquantel and artemisinin derivatives: *in vitro* and *in vivo* investigations with adult *Echinostoma caproni*. Antimicrob Agents Chemother. 2006c; 50, 803-805.

Keiser J, Utzinger J, Vennerstrom JL, Dong Y, Brennan G, Fairweather I. Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. Trans R Soc Trop Med Hyg. 2007; 101, 1219-1222.

Keiser J, Rinaldi L, Veneziano V, Mezzino L, Tanner M, Utzinger J, Cringoli G. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res. 2008; 103, 517-522.

Keiser J, Utzinger J. Food-borne trematodiases. Clinical Microbiol Rev 22, 466-483.
Keiser J. 2010. *In vitro* and *in vivo* trematodes models for chemotherapy studies. Parasitology. 2009; 137, 589-603.

Keiser J, Morson G. *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Exp Parasitol. 2008; 118, 228-237.

Kim CS, Min DY. Synthesis of praziquantel derivatives and their *in vitro* activity against adult *Clonorchis sinensis*. Arch Pharm Res. 1998; 21, 744-8.

Klayman, DL. Qinghaosu (artemisinin): an antimalarial drug from China. Science. 1985; 228, 1049–55.

Koné WM, Vargas M, Keiser J. Anthelmintic activity of medicinal plants used in Côte d'Ivoire for treating parasitic diseases. Parasitol Res. 2012; 110, 2351–2362

Kostadinova A, Gibson DI. The systematic of the Echinostomes. In Echinostomes as experimental models for biological research, B. Fried and J. K. Graczyk (eds.). Kluwer, Dordrecht, The Netherlands . 2000; p. 31–57.

Krautz-Peterson G, Ndegwa D,Vasquez K, Korideck H, Zhang J, Peterson JD, Skelly PJ. Imaging schistosomes *in vivo*. The FASEB J. 2009; 23, 2674-2677.

Krishna S, Bustamante L, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. Trends Pharm Sci. 2008; 29, 520-527.

Kruse DM, Hosier DW, Fried B. The expulsion of *Echinostoma trivolvis* (Trematoda) from ICR mice: scanning electron microscopy of the worms. Parasitol Res. 1992; 78, 74-77.

Kunz WSchistosome male-female interaction: induction of germ-cell differentiation. Trends Parasitol. 2001;17, 227-31.

 Kusel JR, Al-Adhami BH, Doenhoff MJ. The schistosome in the mammalian host: understanding the mechanisms of adaptation. Parasitology. 2007; 134, 1477-526.

Lackie AM. The activation of infective stage of endoparasites and vertebrates. Biological reviews 50. 1975; 285-323.

Le W, You J, Yang Y, Mei J, Guo H, Yang H, Zang C. Studies on the efficacy of artemether in experimental schistosomiasis. Acta Pharm Sin. 1982; 17, 187–93.

Le WJ, You JQ, Mei JY. Chemotherapeutic effect of artesunate in experimental schistosomiasis. Acta Pharm. Sin. . 1983;18: 619 –21.

Leles D, Cascardo P, Friere Ados S, Maldonado A, Sianto L, Araújo A. Insight about echinostomiasis by paleomolecular diagnosis. Parasitol Inter. 2014; 63, 646-649.

Li W, Mo W, Shen D, Sun L, Wang J, Lu S, Gitschier JM, Zhou B. Yeast model uncovers dual roles of mitochondria in action of artemisinin. PLoS Genet. 2005; 1: e36.

Lie KJ and Basch PF. The life history of *Echinostoma paraensei* sp. n. (Trematoda: Echinostomatidae). J Parasitol. 1967; 53:1192-1199.

Liu R, Dong HF, Jiang MS. Artemisinin: the gifts from traditional Chinese medicine not only for malaria control but also for schistosomiasis control. Parasitol Res. 2012; 110, 2071–2074.

Liu YX, Wu W, Liang Y J, Jie ZL, Huang YX. New uses for Old Drugs: The tale of artemisinin derivatives in the elimination of schistosomiasis japonica in China. Molecules. 2014; 19, 15058-15074.

Lopes Torres, EJ, De Souza W, Miranda K. Comparative analysis of Trichuris muris surface using conventional, low vacum, environmental and field emission scanning electron microscopy. Vet Parasitol. 2013; 196, 409-416.

Lorsuwannarat N, Piedrafita D, Chantree P, Sansri V, Songkoomkrong S, Bantuchai S, Sangpairot K, Kueakhai P, Changklungmoa N, Chaichanasak P, Chansela P, Sobhon P. The *in vitro* anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*. Exp Parasitol. 2014; 136, 5-13.

Lovis L, Mak TK, Phongluxa K, Ayé Soukhathammavong P, Vonghachack Y, Keiser J, Vounatsou P, Tanner M, Hatz C, Utzinger J, Odermatt P, Akkhavong K. Efficacy of praziquantel against *Schistosoma mekongi* and *Opisthorchis viverrini*: a randomized, single-blinded dose-comparison trial. PLoS Negl Trop Dis. 2012; 6:e1726.

Luo XD, Shen CC. The Chemistry, Pharmacology, and Clinical Applications of Qinghaosu (Artemisinin) and its derivatives. Med Res Rev. 1987; 7, 29-52.

Machado-Silva JR, Pelajo-Machado M, Lenzi HL, Gomes DL. Morphological study of adult male worms of *Schistosoma mansoni* by confocal laser scanning microscopy. Mem Inst Oswaldo Cruz. 1998; 93, 303-307.

Mackiewicz JS, Ehrenpris MB. Calcareous corpuscles distribution in caryophillid cestode: possible evidence of cryptic segmentation. Proc Helminthol Soc Wash. 1980; 47, 1-9.

Mahler H, Christensen NO, Hindsbo O. Studies on the reproductive capacity of *Echinostoma caproni* (Trematoda) infections in NMRI mice. Proc Helminthol Soc Wash. 1995; 55, 265-269.

Majima M. On *Echinostoma macrorchis* in man. Ibid . 1927; 2552, 2260-2263.

Maldonado A, Loker ES, Morgan JAT, Lanfredi RM. Description of the adult worms of a new Brazilian isolate of *Echinostoma paraensei* (Platyhelminthes: Digenea) from its natural vertebrate host *Nectomys squamipes* by light and scanning electron microscopy and molecular analysis. Parasitol Res. 2001a; 87, 840-848.

Maldonado A, Vieira GO, Garcia JS, Rey L, Lanfredi RM. Biological aspects of a new of isolate of *Echinostoma paraensei* (Trematoda: Echinostomatidae): susceptibility of sympatric snails and the natural vertebrate host. Parasitol Res. 2001b; 87: 853-859.

Maldonado A, Vieira GO, Lanfredi RM. *Echinostoma luisreyi* n. sp. (Platyhelminthes: Digenea) by light and scanning electron microscopy. J Parasitol. 2003; 89, 800–808.

Maldonado A, Zeitone BK, Amado LA, Rosa IF, Machado-Silva JR, Lanfredi RM. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. J Helminthol. 2005; 79, 345-351.

Maldonado A, Lanfredi RM. Echinostomes in the wild. In: Toledo R, Fried B. (Eds.), The Biology of Echinostomes - from the Molecule to the Community. Springer, New York, . 2009; 129–145.

Manger PM, Fried B. Infectivity, growth and distribution of preovigerous adults of *Echinostoma caproni* in ICR mice. J. Helminthol. 1993; 67, 158-160.

Martínez C, Paredes R, Stock RP, Saralegui A, Andreu M, Cabezón C, Ehrlich R, Galanti N Cellular organization and appearance of differentiated structures in developing stages of the parasitic platyhelminth *Echinococcus granulosus*. J Cell Biochem . 2005; 94,327-35.

McIntosh HM, Olliaro P. Artemisinin derivatives for treating severe malaria. Cochrane Database Syst Rev. 2000; CD000527.

Mehlhorn H, Becker B, Andrews P, Thomas H, Frenkel JR. *In vivo* and *in vitro* experimentas on the effects of praziquantel on *Schistosoma mansoni*: a light and electron microscopy study. Arzneimittelforchiing. 1981; 31, 544-554.

Meshnick SR, Thomas A, Ranz A, Xu CM, Pan HZ. Artemisinin (quinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. Mol Biochem Parasitol. 1991; 49, 181-189.

Meshnick SR, Jefford CW, Posner GH, Avery MA, Peters W. Second-generation antimalarial endoperoxides. Parasitol Today. 1996;12, 79-82.

Meshnick SR, Taylor TE, Kamchonwongpasan S. Artemisinin and antimalarial endoperoxide: from herbal remedy to targeted chemotherapy. Microbial Rev. 1996; 60, 301-315.

Moll L, Gaasenbeek CP, Vellema P, Borgsteede FH. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The netherlands. Vet Parasitol. 2000; 91, 153-8.

Mohandas A, Nadakal AM. In vivo development of *Echinostoma malayanum* Leiper, 1911 with notes on effects of population density, chemical composition and pathogenicity and in vitro excystment of the metacercariae (Trematoda: Echinostomatidae) Z. Parasitenkd. 1978; 55, 139-151.

Muñoz-Antoli C, Cortés A, Torres D, Esteban JG, Toledo R. Haematological changes in the laboratory rat *Rattus norvegicus* infected with *Echinostoma caproni* (Trematoda: Echinostomatidae). J Helminthol. 2014; 3,1-5.

Ndjonka D, Rapado LN, Silber AM, Wrenger C. Natural products as a source for treating neglected parasitic diseases. Int. J.Mol. Sci. 2013; 14, 3395-3439.

NevesJKAL, Botelho SPS, Melo CML, Pereira VRA, Lima MCA, Pitta IR, Albuquerque MCPA, Galdino SL. Biological and immunological activity of new imidazolidines against adult worms of *Schistosoma mansoni.* Parasitol Res. 2010; 107, 531-538.

Neves RH, Pereira MJS, Gomes DC, Oliveira RMF, Machado-Silva JR. Morphometric differences of adult worms from sympatric samples of *Schistosoma mansoni* Sambon, 1907 isolated from rodents and humans. Mem Inst Oswaldo Cruz. 1998; 93, 309-312.

Neves RH, Biolchini CL, Machado-Silva JR, Carvalho JJ, Branquinho TB, Lenzi HL, Hulstijn M, Gomes DC. A new description of the reproductive system of *Schistosoma*

mansoni (Trematoda: Schistosomatidae) analyzed by confocal laser scanning microscopy. Parasitol Res. 2005; 95, 43-49.

Nollen PM. Mating behaviour of *Echinostoma caproni* and *E. trivolvis* in concurrent infections in hamsters. Int J Parasitol. 1997; 27(1):71-5.

Ohyama F. Effect of acid pepsin pretreatment, bile acids and reductants on the excystation of *Clonorchis sinensis* (Trematoda: Opisthorchiidae) metacercariae *in vitro*. Parasitol Int. 1998; 47, 29-39.

O'Neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin – The date continues. Moleculate. 2010; 15, 1705-1721.

Overend DJ, Bowen FL. Resistance of *Fasciola hepatica* to triclabendazole. Aust Vet J . 1995; 72, 275-276.

Panic G, Ingram K, Keiser J. Development of an *in vitro* drug sensitivity assay based on newly excysted larvae of *Echinostoma caproni*. Parasit Vectors. 2013; 6, 237–245.

Pawlowski ID, Yap KW, Thompson RC. Observations on the possible origin, formation and structure of calcareous corpuscles in taeniid cestodes. Parasitol Res. 1988; 74, 293-296.

Pinheiro J, Maldonado A, Lanfredi RM. Light and scanning electron microscopy of the miracidium of *Echinostoma paraensei* (Trematoda, Echinostomatidae). Vet Parasitol. 2004a; 121, 265-75.

Pinheiro J, Maldonado AJ, Attias M, Lanfredi RM. Morphology of the rediae of *Echinostoma paraensei* (Trematoda: Echinostomatidae) from its intermediate host *Lymnaea columella* (Mollusca, Gastropoda). Parasitol Res. 2004b; 93, 171-7.

Pinheiro P, Maldonado A. Attias M, Landrefi RM. Ultraestruture of the miracidium of *Echinostoma paraensei* Lie and Basch, 1967 (Trematoda: Echinostomatidae). Parasitol Res. 2005; 97:367-372.

Pinheiro P, Maldonado A, Lanfredi RM. Physiological changes in *Lymnaea columella* (Say, 1817)(Mollusca, Gastropoda) in response to *Echinostoma paraensei* Lie and Basch, 1967 (Trematoda: Echnostomatidae). Parasitol Res. 2009; 106, 55-59.

Posner GH, Meshnick SR. Radical mechanism of action of the artemisisnin type compounds.Trends Parasitol. 2001; 17, 266-267.

Price RN. Artemisinin drugs: novel antimalarial agents. Expert Opin. Investig. Drugs. 2000; 9, 1815–27.

Riper CL. Praziquantel and *Fasciola hepatica* infection. Trans R Soc Trop Med Hyg. 1990; 84, 610.

Robert A, Benoit-Vical F, Claparols C, Meunier B. The antimalarial drug artemisinin alkylates heme in infected mice. Proc. Natl. Acad. Sci. USA. 2005; 102 , 13676–13680.

Rodrigues JJ, Ferreira HB, Farias SE, Zaha A. A protein with a novel calcium-binding domain associated with calcareous corpuscles in *Echinococcus granulosus*. Biochem Biophys Res Commun. 1997; 237, 451-456.

Rossi ML, Balfour CD, Fried B. Excystation and infectivity of *Echinostoma caproni* metacercariae after prolonged stored. Comp. Parasitol. 2001; 68, 126-129.

Ross F, Hernández P, Porcal W, López GV, Cerecetto H, González M, Basika T, Carmona C, Fló M, Maggioli G, Bonilla M, Gladyshev VN, Boiani M, Salinas G. Identification of thioredoxin glutathione reductase inhibitors that kill cestode and trematode parasites. PLoS One. 2012; 7, e35033.

Russell WMS, Burch RL. The Principles 0f Humane Experimental Technique. London: Methuen and Co. Ltd. 1959

Saxton TM, Fried B, Peoples RC. Excystation of the metacercariae of *Echinostoma trivolvis* and *Echinostoma caproni* in a trypsin–bile salts–cysteine medium and morphometric analisys of the larvae. J Parasitol. 2008; 94, 669–671.

Šebelová S, Sterwart MT, Mousley A, Fried B, Marks JN, Halton DW. The musculature and associated innervation of adult and intramolluscan stages of *Echinostoma caproni* (Trematoda) visualized by confocal microscopy. Parasitology Research. 2004; 93, 196-206.

Seo, BS Chun, KS, Chai, JY, Hong, SJ, Lee, SH. Studies on intestinal trematodes in Korea XVII. Development and egg laying capacity of *Echinostoma hortense* in albino rats and human experimental infection. J Parasitology. 1985; 23, 24–32.

Shaw MK, Erasmus DA. *Schistosoma mansoni*: structural damage and tegumental repair after in vivo treatment with PQZ. Parasitol. 1987; 94, 243-54.

Shuhua X, Binggui S, Utzinger J, Chollet J, Tanner M. Transmission electron microscopic observations on ultrastructural damage in juvenile *Schistosoma mansoni* caused by artemether. Acta Trop. 2002; 81, 53–61.

Slais J. Functional morphology of cestode larvae In: Dawnes B (ed). Adv. Parasitol. Academic Press, New York. 1973; 396-466.

Sianto L, Reinhard KJ, Chame M, Chaves S, Mendonça S, Gonçalves ML, Fernades A, Ferreira LF, Araújo A. The finding of Echinostoma (Trematoda: Digenea) and hookworm eggs in coprolites collected from a Brazilian mummified body dated 600-1,200 years before presente. J Parasitol. 2005; 91, 972-975.

Silva-Leitão FW, Biolchini CL, Neves RH, Machado-Silva JR. Development of *Schistosoma mansoni* in the laboratory rat analyzed by light and confocal laser scanning microscopy. Exp Parasitol. 2009; 123, 292-295.

Smith SA, Richards KS. Utrastructure and microanalyses of the calcareous corpusclesof the protoscolices of *Echinococcus granulosus*. Parasitol Res. 1993; 79, 245-250.

Smits HL. Prospects for the control of neglected tropical diseases by mass drug administration. Expert Rev Anti Infect Ther. 2009; 7, 37-56.

Smoluk JL, Fried B. Light microscopy observation of *Echinostoma revolutum* (Trematoda) metacercariae during *in vitro* excystation. Parasitol Res. 1994; 80, 435–438.

Smyth JD. The physiology of cestodes. Freeman and Company. San Francisco
Sotillo J, Trudgett A, Halferty L, Marcilla A, Esteban JG, Toledo R. 2010. *Echinostoma caproni*: differential tegumental responses to growth in compatible and less compatible hosts. Exp Parasitol. 1969; 125, 304-9.

Souza JGR, Garcia JS, Manso PPA, Neves RH, Maldonado A, Machado-Silva JR. Development of the reproductive system of the *Echinostoma paraensei* in *Mesocricetus auratus* analyzed by light and confocal scanning laser microscopy. Exp Parasitol. 2011; 128, 341-346.

Souza JG, Garcia J, Neves RH, Machado-Silva JR, Maldonado A. *In vitro* excystation of *Echinostoma paraensei* (Digenea: Echinostomatidae) metacercariae assessed by light, morphometry and confocal laser scanning microscopy. Exp Parasitol. 2013; 135, 701-707.

Stein PC, Basch PF. Metacercarial cyst formation *in vitro* of *Echinostoma paraensei*. J Parasitol. 1977; 63, 1031-1040.

Sukhdeo MVK, Mettrick DF. The behavior of juvenile *Fasciola hepatica* . J Parasitol. 1987; 72, 492-497.

Tansatit T, Sahaphong S, Riengrojpitak S, Viyanant V, Sobhon P. *Fasciola gigantica*: The *in vitro* effects of artesunate as compared to triclabendazole on the 3-weeks-old juvenile. Exp. Parasitol. 2012; 131, 8-19.

Toledo R. Espert A. Muñoz-Tunholi C. Marcilla A Fried B. Estebam JG. Development of antibody –based capted enzyme-linked immunosorbent assay for detecting *Echinostoma caproni* (Trematoda) in experimentally infected rat: kinetic of coprantigen excretion. J Parasitol. 2003; 89, 1227-1231.

Toledo R, Espert A, Carpena I. The comparative development of *Echinostoma caproni* (Trematoda: Echinostomatidae) adults in experimentally infected hamsters and rats. Parasitol. Res. 2004a; 93, 439-444.

Toledo R, Espert A, Muñoz-Antoli C, Marcilla A, Fried B, Esteban JC. Kinetic of *Echinostoma caproni* (Trematoda: Echinostomatidae) antigens in feces and serum of experimentally infected hamster and rats. J. Parasitol. 2004b; 90, 752-758.

Toledo R, Fried B. Echinostomes as experimental models for interactions between adult parasites and vertebrate host. Trends Parasitol. 2005; 21:251-254.

Toledo R, Monteagudo C, Espert A, Fried B, Esteban JG, Marcilla A. *Echinostoma caproni*: Intestinal pathology in the golden hamster, a highly compatible host, and the wistar rat, a less compatible host. Exp Parasitol. 2006;112, 164-171.

Trelis M, Sotillo J, Monteagudo C, Fried B, Marcilla A, Esteban JG, Toledo R. *Echinostoma caproni* (Trematoda): differential *in vivo* cytokine responses in high and low compatible hosts. Exp Parasitol. 2011; 127, 387-97.

Tunholi VM, Lustrino D, Tunholi-Alves VM, Mello-Silva CC, Maldonado A, Rodrigues MLA, Pinheiro JS. Changes in the reproductive biology of *Biomphalaria glabrata* infected with differente doses of *Echinostoma paraensei* miracidia. Journal of Invetebrate Pathology. 2011a106, 192-195.

Tunholi VM, Lustrino D, Tunholi-Alves VM, Mello-Silva CC, Rodrigues MLA, Maldonado A, Pinheiro JS. Biochemical profile of *Biomphalaria glabrata* (Mollusca: Gastropoda) after infection by *Echinostoma paraensei* (Trematoda: Echinostomatidae). Parasitology Resesrach. 2011b; 109, 885-891.

Tunholi VM, Tunholi-Alves VM, Lustrino, Golo OS, Maldonado A, Bittencourt V, Rodrigues MLA, Pinheiro JS. Lipid levels in *Biomphalaria glabrata* infected with different doses of *Echinostoma paraensei* of miracidia. Experimental Parasitology 2011c; 128, 212-216.

Ursone R, Fried B. Light and scanning electron microscopy of *Echinostoma caproni* (Trematoda) during maturation in ICR mice. Parasitol Res. 1995; 81, 45-51.

Utzinger J, Xiao SH, Tanner M, Keiser J. Artemisinins for schistosomisasis and beyond. Curr Opin Investig Drugs. 2007; 105-116.

Valero MA, Darce NA, Panova M, Mas-Coma S. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. Vet Parasitol. 2001; 102, 85-100.

Van Agtmael MA, Eggelte TA, van Boxtel CJ. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends Pharmacol Sci. 1999; 20, 199 –205.

Vargas-Parada L, Lanclette JP. Role of the calcareous corpuscles in cestode physiology: a review. Rev. Latinoam Microbiol. 1999; 41, 303-307.

Vargas-Parada L, Merchant MT, Willms K, Laclette JP. Formation of calcareous corpuscles in the lumen of excretory canals of *Taenia solium cysticerci*. Parasitol Res. 1999; 85, 88-92

Vroman JA, Alvim-Gaston M, Avery MA. Current progress in the chemistry, medicinal chemistry and drug design of artemisinin based antimalarials. Curr. Pharm. Des. 1999; 5, 101–38.

Wang CC, Statish RL. Pancreatic chymotrypsin as the essential enzyme for excystation of *Eimeria tenella*. J Parasitol. 1975; 61, 923-927.

Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, Zhou B. Artemisinin directly targets malarial mitochondria through its specific m itochondrial activation. PLoS One. 2010; 5:e9582.

Weinstein MS, Fried B. The expulsion of *Echinostoma trivolvis* and retention of Echinostoma caproni in the ICR mouse: pathological effects. Int J Parasitol. 1991 21, 255-257.

Who. The Control of Schistosomiasis: Report of a WHO Expert Committee. World Health Organization, Geneva. 1985

Xiao SH, Catto BA, Webster LT. Effects of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. J Infect Dis. 1985; 151, 1130–7.

Xiao S, Catto BA. *In vitro* and *in vivo* studies of the effect of artemether on *Schistosoma mansoni*. Antimicrob Agents Chemother. 1989; 1557-1562.

Xiao SH, Yin JW, Mei JY, You JQ, Li Y, Jiang HJ. Effect of arteether on *Schistosoma japonicum*. Acta Pharm. Sin. 1992; 27, 161 –5.

Xiao SH, Shen BG, Horner J, Catto BA. 1996b. Tegument changes of *Schistosoma japonicum* and *Schistosoma mansoni* in mice treated with artemether. Acta Pharmacol Sin 17, 535 –7.

Xiao SH, You JQ, Mei JY, Guo HF, Jiao PY, Sun HL, Yao MY, Feng Z. Effect of artemether on glucose uptake and glycogen content in *Schistosoma japonicum*. Acta Pharmacol Sin. 1997; 18, 363-367.

Xiao SH, You JQ, Guo HF, Jiao PY, Mei JY, Yao MY, Feng Z. Effect of artemether on glyceraldehydes-3-phosphate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase of *Schistosoma japonicum* harbored in mice. Acta Pharmacol Sin. 1998a; 19, 279-81.

Xiao SH, You JQ, Guo HF, Jiao PY, Mei JY, Yao MY, Feng Z. Effect of artemether on hexokinase glucose phosphate isomerase and phosphofructokinase of *Schistosoma japonicum* harbored in mice. Chin. J. Parasitol. Para. Dis. 1998b; 16, 25-28.

Xiao SH, You JQ, Guo HF, Mei JY, Jiao PY, Yao MY, Zhuang ZN, Feng Z. Effect of artemether on phosphorylase, lactate dehydrogenase, adenosine triphosphatase, and glucosephosphate dehydrogenase of *Schistosoma japonicum* hoarnored in mice. Acta Pharmacol Sin. 1999; 20, 750-754.

Xiao SH, Booth M, Tanner M. The prophylactic effects od artemether against *Schistosoma japonicum* infection. Parasitol Today. 2000a; 16, 122-126.

Xiao SH, Chollet J, Weiss NA, Bergquist RN, Tanner M. Preventive effect of artemether in experimental animals infected with *Schistosoma mansoni*. Parasitol Int. 2000b; 49, 19 –24.

Xiao SH, Hotez PJ, Tanner M. Artemether, an effective new agent for chemoprophylaxis against schistosomiasis in China: its *in vivo* effect on the biochemical metabolism of the asian schistosome. Southeast Asian J Trop Med Public Health. 2000c; 31, 724–32.

Xiao SH, Shen BG, Chollet J, Tanner M. Tegumental changes in 21-day-old *Schistosoma mansoni* harboured in mice treated with artemether. Acta Trop. 2000d; 75, 341–348.

Xiao SH, Shen BG, Chollet J, Utzinger J, Tanner M. tegumental alterations in juvenile *Schistosoma haematobium* harbored in hamsters following artemether treatment. Parasitol Int. 2001b; 50, 175-183.

Xifeng T, Baodong Z. Ultrastructural observation on the formation and metabolism of calcareous corpuscles in *Cysticercus cellulosae*. Chin J Parasitol Parasitic Dis. 1998; 16, 364-367.

Yang HJ. Immunoblot findings of calcareous corpuscles binding proteins in cyst fluid of *Taenia solium* metacestodes. Korean J Parasito. 2004; 42, 141-143.

Yao G, Huffman JE, Fried B. 1991. The effects of crowding on adults of *Echinostoma caproni* in experimentally infected golden hamsters. J Helmintol 65, 248–254.

Yin JW, Yang YQ, Xiao SH, Li Y, Jiang HJ. Comparative studies on histological and histochemical alterations of *Schistosoma japonicum* induced by arteether and artemether. Acta Pharmacol Sin. 1991; 12, 478–80.

You JQ, Guo H, Mei JY, Jiao P, Feng J, Yao M, Xiao SH. Effect of artemether on glycogen, protein, alkaline phsphatase and acid phosphatase of *Schistsoma japonicum*. Chin J Parasitol Para Dis. 1994a; 12, 275-278.

Yue WJ, You JQ, Mei JY. Effects of artemether on *Schistosoma japonicum* adult worms and ova. Acta Pharmacol Sin. 1984; 5, 60 –63.

Ziffer H, Highet RJ, Klayman DL. Artemisinin: an endoperoxidic antimalarial from *Artemisia annua L*. Prog. Chem. Org. Nat. Prod. 1997;72: 121–214.