UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

AÇÃO DO ÓLEO ESSENCIAL DE *Hyptis mutabilis* EM *Rhamdia quelen* PARASITADOS COM *Ichthyophthirius multifiliis* E SEUS EFEITOS EM PEIXES SADIOS

DISSERTAÇÃO DE MESTRADO

Jessyka Arruda da Cunha

Santa Maria, RS, Brasil

2015

AÇÃO DO ÓLEO ESSENCIAL de *Hyptis mutabilis* EM *Rhamdia quelen* PARASITADOS COM *Ichthyophthirius multifiliis* E SEUS EFEITOS EM PEIXES SADIOS

Jessyka Arruda da Cunha

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia**

Orientador: Profa. Dra. Berta Maria Heinzmann

Santa Maria, RS, Brasil

2015

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Cunha, Jessyka Arruda da
Ação do óleo essencial de Hyptis mutabilis em Rhamdia quelen parasitados com Ichthyophthirius multifiliis e seus efeitos em peixes sadios / Jessyka Arruda da Cunha.-2015.
75 f.; 30cm

Orientadora: Berta Maria Heinzmann
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós-Graduação em Farmacologia, RS, 2015

1. Aquicultura Orgânica 2. Controle Biológico 3. (-)-globulol 4. Doença Parasitária 5. Peixe I. Heinzmann, Berta Maria II. Título.

© 2015
Todos os direitos autorais reservados a Jessyka Arruda da Cunha. A reprodução de partes ou do todo deste trabalho só poderá ser feita mediante a citação da fonte.
E-mail: jessyka.arruda.c@hotmail.com

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Farmacologia

A comissão examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

AÇÃO DO ÓLEO ESSENCIAL de *Hyptis mutabilis* EM *Rhamdia quelen* PARASITADOS COM *Ichthyophthirius multifiliis* E SEUS EFEITOS EM PEIXES SADIOS

elaborada por
Jessyka Arruda da Cunha

Como requisito parcial para obtenção do grau de
Mestre em Farmacologia

Comissão Examinadora:

Berta Maria Heinzmann, Dra. (UFSM)
(Orientadora)

Luciano de Oliveira Garcia, Dr. (FURG)

Mauro Alves da Cunha, Dr. (UFSM)

Santa Maria, 25 de Fevereiro de 2015.

AGRADECIMENTOS

Agradeço...

Agradeço à minha família e meu namorado, pelo apoio em minhas decisões, além de ter dado todo o suporte necessário para que eu pudesse conquistar meus sonhos.

À Universidade Federal de Santa Maria e ao Programa de Pós Graduação em Farmacologia.

A Professora Berta Maria Heinzmann, pela orientação, atenção dispensada, paciência, dedicação, amizade, profissionalismo e confiança.

Aos Professores Bernardo Baldisserotto e Rodrigo de Almeida Vaucher pela dedicação, co-orientação e profissionalismo.

Aos colegas do Laboratório de Extrativos Vegetais e Laboratório de Fisiologia de Peixes que fizeram e fazem parte da minha trajetória na pós-graduação, em especial ao colega Fernando Jonas Sutili pela amizade, competência, paciência e ensinamentos passados e as alunas de graduação Anita Mota e Cecília Scheeren pela amizade e auxilio no decorrer do trabalho.

RESUMO

Dissertação de Mestrado
Programa de Pós–Graduação em Farmacologia
Universidade Federal de Santa Maria

AÇÃO DO ÓLEO ESSENCIAL de *Hyptis mutabilis* EM *Rhamdia quelen* PARASITADOS COM *Ichthyophthirius multifiliis* E SEUS EFEITOS EM PEIXES SADIOS

AUTOR: JESSYKA ARRUDA DA CUNHA
ORIENTADORA: BERTA MARIA HEINZMANN
Data e Local da Defesa: Santa Maria, 25 de Fevereiro de 2015.

O objetivo deste estudo foi avaliar a atividade do óleo essencial (OE) de folhas de *Hyptis mutabilis*, bem como o seu principal componente (-)-globulol contra o parasito de peixe *Ichthyophthirius multifiliis* (Ich) e os seus efeitos sobre parâmetros hematológicos, bioquímicos e imunológicos em jundiá (*Rhamdia quelen*). No primeiro experimento, peixes infectados naturalmente foram tratados com óleo essencial (0, 10 e 20 $mg.L^{-1}$ e 199,26 $\mu L.L^{-1}$ etanol) por quatro dias através de diferentes métodos de exposição (uma única aplicação no início do experimento, duas vezes com um intervalo de 48 h e banhos diários de 1h). A sobrevivência dos peixes e o número de parasitos (trofontes por peixe) foram avaliados após 48 e 96 h. Os banhos diários de 1h apresentaram a melhor sobrevivência, quando comparados com uma única aplicação e duas aplicações com intervalo de 48 h, e essa metodologia foi definida para o segundo experimento, em que animais infectados foram expostos a (-)-globulol em 2,5 e 5 $mg.L^{-1}$. As concentrações mais eficazes nos experimentos 1 e 2 foram escolhidas para experimento 3, no qual animais saudáveis foram submetidos a banhos diários de 1h com OE (20 $mg.L^{-1}$) ou (-)-globulol (2,5 $mg.L^{-1}$). Quatro dias mais tarde, os animais tiveram parâmetros hematológicos, bioquímicos, e imunológicos avaliados. Após a exposição de animais saudáveis ao OE, foi detectado um aumento significativo no hematócrito (28,42%) e de número total de eritrócitos (2,11 x 10^6 mL^{-1}) e leucócitos (48,82 x 10^3 mL^{-1}), enquanto a contagem total de trombócitos (20,50 x 10^3 μL^{-1}) diminuiu em relação a estudos anteriores com jundiá. No entanto, a exposição ao (-)-globulol aumentou apenas o número de leucócitos (70,87 x 10^3 mL^{-1}), relativo aos valores padrão já reportados a esta espécie. O colesterol total mostrou níveis elevados (226,66 $mg.dL^{-1}$) em peixes expostos ao OE, enquanto que o grupo tratado com (-)-globulol apresentou maior valor para LDL (244,66 mg dL^{-1}) e inferior para HDL (87,66 $mg.dL^{-1}$) do que os grupos de controle e etanol. OE e (-)-globulol aumentaram a sobrevida dos peixes infectados com ich e alteraram alguns parâmetros hematológicos e bioquímicos, tais como albumina, colesterol total, HDL, LDL, hematócrito, eritrócitos, leucócitos, trombócitos e MCV. Nenhuma diferença significativa foi detectada em ensaio imune inespecífica entre os grupos tratados e controle, indicando que o OE e (-)-globulol não aumentaram a imunidade inata do peixe.

Palavras-chave: Aquicultura Orgânica. Controle Biológico. (-)-globulol. Doença Parasitária. Peixe.

ABSTRACT

Master Dissertation
Post-Graduate Course in Pharmacology
Universidade Federal de Santa Maria

ACTION OF *Hyptis mutabilis* ESSENTIAL OIL IN *Rhamdia quele*) PARASITIZED WITH *Ichthyophthirius multifiliis* AND ITS EFFECT IN HEALTHY FISH

AUTHOR: JESSYKA ARRUDA DA CUNHA
ADVISOR: BERTA MARIA HEINZMANN
Date and place of the defense: Santa Maria, February 25th, 2014.

The aim of this study was to evaluate the activity of leaves essential oil (EO) of *Hyptis mutabilis*, as well as its major constituent (-)-globulol against the fish parasite *Ichthyophthirius multifiliis* (Ich) and their effects on hematological, biochemical and immunological parameters in silver catfish (*Rhamdia quelen*). In the first experiment lasting four days, naturally infected fish were treated with EO (0, 10 and 20 $mg.L^{-1}$ and 199.26 $\mu L.L^{-1}$ ethanol) by means of different exposure methods (a single application at the beginning of the experiment, twice with a 48 h interval, and 1 h daily baths). Fish mortality and the number of parasites (trophonts per fish) were assessed after 48 and 96 h. The 1 h daily baths provided the best survival when compared to a single application and two applications with a 48 h interval, and this methodology was set for the second experiment, in which infected animals were exposed to (-)-globulol at 2.5 and 5 $mg.L^{-1}$. The most effective concentrations in the experiments 1 and 2 were chosen for experiment 3, in which healthy animals were subjected to 1 h daily baths with EO (20 $mg.L^{-1}$) or (-)-globulol (2.5 $mg.L^{-1}$). Four days later, the animals had hematological, biochemical, and immunological parameters assessed. After exposure of healthy animals to EO, significant increase was detected in hematocrit (28.42 %), erythrocytes (2.11 10^6 μL^{-1}) and leukocytes (48.82 10^3 μl^{-1}), while the total count of thrombocytes (20.50 10^3 μL^{-1}) decreased in comparison to previous studies with *Rhamdia quelen*. However exposure to (-)-globulol increased only the leukocyte number (70.87 10^3 μl^{-1}) related to the default values already reported to this species. Total cholesterol showed elevated levels (226.66 mg dL^{-1}) in fish exposed to EO, while the group treated with (-)-globulol presented higher LDL (244.66 mg dL^{-1}) and lower HDL (87.66 mg dL^{-1}) values than the control and ethanol groups. EO and (-)-globulol increased survival of fish infected with ich and altered some hematological and biochemical parameters, such as such as albumin, total cholesterol, HDL, LDL, hematocrit, erythrocytes, leucocytes, thrombocytes and MCV. No significant differences were detected in nonspecific immune assay between the treated groups and control, indicating that the EO and (-)-globulol did not increase innate immunity of fish.

Key-words: Organic Aquaculture. Biological Control. (-)-globulol. Parasitic Disease.Fish.

LISTA DE TABELAS

MANUSCRITO

LISTA DE ILUSTRAÇÕES

MANUSCRITO

LISTA DE ANEXOS

SUMÁRIO

INTRODUÇÃO

Na piscicultura, atualmente o Brasil é o país que apresenta o maior potencial de produção de pescado devido ao clima propício, bem como em decorrência da rede hidrográfica que contém aproximadamente 12% da água doce disponível no planeta (CONROY, 1975; GUEDES, 1980). Dentro da ordem Siluriforme e da família Heptapteridae, encontra-se a espécie *Rhamdia quelen*, popularmente conhecida como jundiá (SWARÇA *et al*., 2000). Esta espécie animal possui distribuição neotropical e tem despertado interesse crescente devido as suas características de cultivo (BALDISSEROTTO, 2009). Além disso, possui hábitos noturnos podendo ser facilmente manipulada em âmbito laboratorial (SILFVERGRIP, 1996; FRACALOSSI *et al*., 2002).

Entretanto, os jundiás possuem susceptibilidade ao parasito *Ichthyophthirius multifiliis* (Ich) o qual ocasiona moratalidade de até 100% dos animais em um curto período de tempo (GARCIA, *et al*., 2007,). Embora para o Brasil não tenham sido encontradas estatísticas informando sobre o montante do prejuízo provocado por este protozoário ciliado, o mesmo causa perdas consideráveis na piscicultura mundial, sendo responsável pelo decréscimo de até 140 mil dólares por ano na economia Europa (DICKERSON, 2012). Além disso, o Ich é facilmente estimulado à proliferação na temperatura ambiente, uma vez que a 20°C o ciclo de vida deste parasito é completado em sete dias (FAO, 2011; DICKERSON, 2012; MATTHEWS, 2005).

A utilização de óleos essenciais que são fonte de compostos farmacologicamente ativos, vem sendo uma alternativa promissora frente aos demais tratamentos já descritos por possuírem vantagens em relação à toxicidade e rentabilidade (CARNEIRO *et al*., 2005; PICÓN-CAMACHO *et al*., 2012). A espécie vegetal *Hyptis mutabilis* (Rich.) Briq. possui na sua constituição uma ampla gama de metabolitos secundários, com destaque para os terpenoides, incluindo monoterpenoides e sesquiterpenoides que compõe o óleo essencial presente tanto em folhas quanto em inflorescências (XIMENES *et al*., 2013).Portanto, este estudo teve como objetivo avaliar a atividade antiparasitária *in vivo* do óleo essencial (OE) de folhas de *Hyptis mutabilis* e de seu constituinte majoritário (-)-globulol na tentativa de controle do parasito *Ichthyophthirius multifiliis* em juvenis de jundiá, através da aplicação de três metodologias distintas. Além disso, foram avaliados parâmetros hematológicos,

bioquímicos e imunológicos de animais saudáveis, submetidos à metodologia que promoveu a maior sobrevivência dos peixes parasitados nos desenhos experimentais 1 e 2 .

1 OBJETIVOS

1.1 Objetivo geral

Avaliar o potencial do óleo essencial (OE) de *Hyptis mutabilis* para o controle de ictiofitiríase na aquicultura.

1.2 Objetivos específicos

Avaliar a atividade antiparasitária *in vivo* do OE de folhas de *Hyptis mutabilis* frente ao *Ichthyophthirius multifiliis* em jundiás;

Avaliar a sobrevivência dos animais expostos ao OE ou ao seu constituinte majoritário, (-)-globulol, em jundiás parasitados com *Ichthyophthirius multifiliis*;

Verificar o potencial de inibição da atividade hemolítica de *A. hydrophila* do OE de *Hyptis mutabilis* e do (-)-globulol utilizando eritrócitos de peixes sadios;

Verificar os efeitos da adição do OE de *Hyptis mutabilis* e do (-)-globulol na água sobre parâmetros bioquímicos e hematológicos em jundiás sadios.

2 REVISÃO BIBLIOGRÁFICA

2.1 Piscicultura no Brasil

O Brasil abriga cerca de 25.000 espécies de peixes de água doce, e em consequência dessa variabilidade de espécies, há uma grande variedade de formas, habitats e características distintas dos animais (CONROY ,VASQUEZ, 1975; GUEDES, 1980).O país produz cerca de 2 milhões de toneladas de pescado, sendo 40% cultivados. Dentre as espécies cultivadas destaca-se a tilápia, que apresentou um aumento de 105% na produção em apenas sete anos (2003-2009) (BRASIL, 2014).

Cada região brasileira produz e cultiva determinadas espécies de peixes, sendo comum a criação de tambaqui e pirarucu na região norte, enquanto que as regiões nordeste e sudeste cultivam principalmente tilápia e camarão-marinho. Já a região sul apresenta cultivos mais diversificados, com carpas, tilápias, jundiás, ostras e mexilhões. Por outro lado, cultivos de tambaqui, pacu e pintado são frequenes na região centro-oeste (BRASIL, 2014).

Segundo os dados disponibilizados pela Agência Nacional de Águas (ANA) em 2010, o Rio Grande do Sul liderava a produção brasileira de pescado, com cerca de 55 mil toneladas. Entretanto, segundo a Emater, em 2013, o volume de pescado somou 17 mil toneladas. Desse total, 9,4 mil foram vendidas, o que gerou um faturamento de R$ 43,9 milhões ao estado, sendo a espécie *Rhamdia quelen* responsável por R$ 636,3 mil desse faturamento (CIGANA, 2014). Dentre os municípios do estado, Vacaria vem sendo um dos maiores produtores de jundiá no RS em sistemas extensivos e semi-intensivos (MANSKE, 2014).

Em contrapartida, a perda exponencial no ramo da aquicultura em decorrência de doenças infecciosas é um aspecto relevante e preocupante. Este fato pode ser atribuído à falta de saneamento básico nas unidades de produção, como a falta de renovação constante ou periódica de água, manejo inadequado, contaminação da água e do solo com herbicidas, além da falta de medidas de prevenção/tratamento frente a parasitoses (KREUTZ *et al*., 2010). De maneira geral, esses fatores alteram direta e indiretamente a resposta imunológica dos peixes, impedindo a supressão à agressão de micro-organismos patogênicos, com destaque para o protozoário ciliado *Ichthyophthirius multifiliis* e a bactéria Gram-negativa *Aeromonas*

hydrophila, contribuindo para um aumento da mortalidade e redução na produtividade (CONROY, VASQUEZ, 1975; KREUTZ *et al*., 2010).

2.2 *Hyptis mutabilis*

A utilização da fitoterapia, que compreende o tratamento de patologias e demais distúrbios através de produtos naturais obtidos de plantas, tem sua referência mais antiga datada há sessenta mil anos (REZENDE, COCCO, 2002). Já na Declaração de Alma-Ata, de 1978, a Organização Mundial da Saúde (OMS) reconheceu que 80% da população dos países em desenvolvimento valia-se de práticas tradicionais nos seus cuidados básicos de saúde e 85% usava extratos vegetais ou preparações a partir dos mesmos (OMS, 1978). Cerca de 50% dos medicamentos aprovados entre 1981 e 2006, pela Food and Drug Administration (FDA), são direta ou indiretamente derivados de produtos naturais, comprovando assim que as plantas medicinais beneficiaram, e continuam beneficiando a humanidade (FERREIRA, PINTO, 2010).

Dentre as espécies medicinais nativas do Brasil destaca-se o gênero *Hyptis* (Lamiaceae), que é formado por aproximadamente 400 espécies distribuídas desde o sul dos Estados Unidos até a Argentina. Esse gênero é composto por ervas, sub-arbustos, arbustos ou árvores pequenas, com caules geralmente quadrangulares e folhas contendo substâncias aromáticas (BORDIGNON, 1990).

No sul do Brasil, pode ser encontrada uma das principais representantes do gênero *Hyptis*, denominada *Hyptis mutabilis* (A. Rich. Briq.). Essa espécie é uma erva anual das Américas, sendo conhecida popularmente como “alfavacão”. Na medicina popular é utilizada para o tratamento de gastrite, dor de cabeça, como expectorante e sedativo, bem como devido aos seus efeitos antimicrobiano, anti-úlcera gástrica, larvicida, antidepressivo, anti-inflamatório e antinociceptivo. Do ponto de vista fitoquímico, essa espécie vegetal possui na sua composição química terpenoides, flavonoides, lactonas, lignanas, alcaloides e óleos essenciais (OE).

Os OEs extraídos desta espécie vegetal possuem como constituintes majoritários terpenoides, que são formados pela condensação de unidades de isopreno (C5). Esses constituintes constituem um dos maiores grupos dentre os metabólitos secundários vegetais,

sendo utilizados tanto na medicina popular quanto na terapêutica por possuírem propriedades sedativas, tranquilizantes e anticonvulsivantes (PASSOS *et al,* 2009). Dentre estes, os derivados monoterpenoides têm demonstrado atividades sobre o SNC, incluindo sedativa, antinociceptiva e antidepressiva (PERGENTINO DE SOUZA *et al.*, 2007). Já os sesquiterpenoides apresentam funções antimicrobianas descritas, além de serem comumente utilizados na indústria farmacêutica como componentes de fragrâncias e cosméticos, produtos de limpeza e aditivos de alimentos (SEO, *et al*., 2008).

O constituinte majoritário dos OE dessa espécie varia de acordo com vários fatores, entre eles o local de coleta e as características genéticas da planta, caracterizando a ocorrência de quimiotipos (FACHINETTO, TEDESCO, 2009; XIMENES *et al*., 2013), bem como de acordo com o órgão vegetal usado para sua extração. A espécie *H. mutabilis* coletada em Santa Maria, RS (Figura 1), forneceu como constituinte majoritário o (-)-globulol, quando o OE foi proveniente de folhas e germacreno D quando o OE foi obtido de inflorescências (SILVA *et al*., 2013).

Figura 1 – Foto da espécie vegetal *Hyptis mutabilis*

Fonte: Acervo da Dra. Lenise Lima

2.3 *Ichthyophthirius multifiliis*

Uma das maiores preocupações na aquicultura é a infecção de espécies de peixes de água doce com o ectoparasito ciliado *Ichthyophthirius multifiliis* Fouquet, 1876. Esse

protozoário é responsável pela patologia ictiofitiríase, conhecida popularmente como “doença dos pontos brancos” devido aos sinais clínicos evidenciados na superfície corpórea dos animais parasitados (BUCHMANN *et al*., 2001; DICKERSON, 2012; MATTHEWS, 2005; BUCHMANN *et al*., 1999; PICÓN-CAMACHO *et al*., 2012; MARTINS *et al*., 2002).

Há relatos na literatura que mencionam que os peixes em algum momento de sua vida irão ter contato com esse parasito, pelo fato do mesmo estar muito difundido nos ambientes de criação ou aquários (MATTHEWS, 2005; BUCHMANN *et al*., 1999). No entanto, o contato não é o principal problema, uma vez que peixes sadios com sistema imunológico imunosuprimido são capazes de combater este parasito e impedir que a infecção ocorra (BUCHMANN *et al*., 2001). Porém, quando os animais passam por situações que possam debilitar seu sistema imunológico como estresse, transporte, alimentação inadequada ou trocas bruscas de temperatura, eles se tornam suscetíveis a infecções oportunistas como esta parasitose (DICKERSON, 2012; MATTHEWS, 2005; POST, 1987; STOSKOPF, 1993; FAIRFIELD, 2000; FAO, 2010; FAO, 2011; CEPAL/ FAO/ IICA, 2011; INFOPESCA, 2012).

O “ich” possui um ciclo de vida bastante complexo, incluindo três estágios que compreendem as fases de teronte, trofonte e tomonte, sendo que em todas elas o parasito apresenta-se sob a forma ciliada. A primeira fase do parasito é denominada teronte, sendo o estágio infectante. Após o protozoário anexar-se à epiderme ou às brânquias do animal, ele se transforma em trofozoíto, sendo que nesta etapa o mesmo aloca-se na superfície do animal, até o momento sadio, e começa a alimentar-se das células do hospedeiro, desencadeando uma irritação cutânea. O parasito perfura e penetra a pele do hospedeiro e se aloja entre a derme e a epiderme. O peixe, por sua vez, em resposta a perfuração/penetração do parasito, desencadeia a restauração da camada perfurada e assim o parasito encistado fica alojado entre estas camadas e inacessível a substâncias externas. Após o amadurecimento do protozoário na pele do peixe, o parasito passa a ser conhecido como trofonte (trofozoíto maduro). Nesta fase o parasito deixa o corpo do animal e fica alocado geralmente no fundo dos aquários e criadouros ou até mesmo aderido à superfície de plantas para completar seu ciclo de vida na água, nesta etapa ocorreo processo de divisão e formação de células-filhas, denominadas tomontes, os quais são móveis. Neste estágio o parasito sobrevive por aproximadamente 48h, quando diferencia-se em teronte e o ciclo se repete (Figura 2) (DICKERSON, 2006,2012; MATTHEWS, 2005; LYNN, 2008; GHIRALDELLI *et al*., 2007; POST, 1987; STOSKOPF,

1993; FAIRFIELD, 2000; FAO, 2010; FAO, 2011; CEPAL/ FAO/ IICA, 2011; INFOPESCA, 2012).

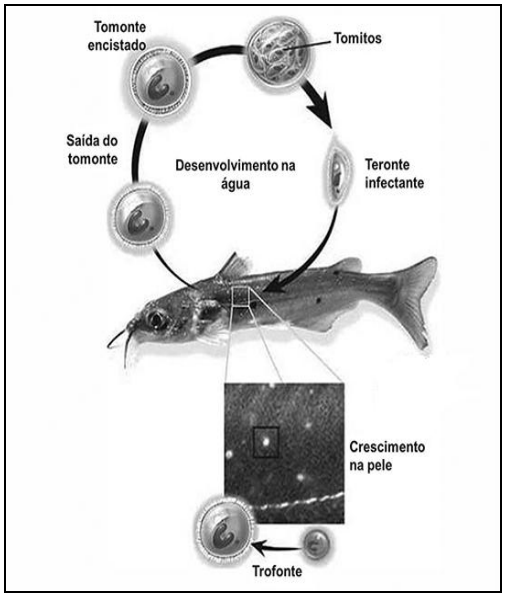


Figura 2 – Representação ilustrativa do ciclo do parasito *Ichthyophthirius multifiliis*

Fonte: Panorama da aquicultura. <http://www.panoramadaaquicultura.com.br/novosite/?p=2120>

Os animais parasitados apresentam pontos brancos e comportamento característico, como desequilíbrio no nado e lesões provocadas pelos próprios animais quando os mesmos debatem-se em pedras ou fundos de aquários, na tentativa de esfregar-se em decorrência da irritação, podendo também desencadear hipóxia, agitação intensa, perda do apetite e eventual morte, por tornarem-se suscetíveis a infecções secundárias, tais como as bacterianas (POST, 1987; STOSKOPF, 1993; FAIRFIELD, 2000; FAO, 2010; FAO, 2011; CEPAL/ FAO/ IICA, 2011; INFOPESCA, 2012).

A solução para controlar o parasito é relativamente fácil quando o tratamento é direcionado para tanques e aquários, sendo realizado conforme as alternativas apresentadas pela literatura, como controle da temperatura e higienização dos taques (FAIRFIELD, 2000). Porém, na maioria das vezes este recurso não se mostra eficaz nas pisciculturas, apenas em âmbito laboratorial ou em aquários. Em vista, das perdas na aquicultura ocasionada pelo Ich, tornou-se necessária a busca pelo tratamento focado no animal e não só nos reservatórios (POST, 1987; STOSKOPF., 1993; FAO, 2010; FAO, 2011; CEPAL/ FAO/ IICA, 2011; INFOPESCA, 2012).

Estudos relatam algumas intervenções frente ao parasito, como a utilização de $4g.L^{-1}$ de sal, o que segundo os autores, melhora a sobrevivência de jundiás parasitados, podendo

ser utilizada então como um tratamento eficaz (GARCIA *et al*., 2007). Contudo, a maioria dos tratamentos disponíveis é a base de produtos químicos de reconhecida toxicidade, administrados na forma de banhos, como formaldeído, o sulfato de cobre, permanganato de potássio, ácidos orgânicos, ferrato de potássio (VI), bronopol e produtos à base de ácido peracético. Entretanto, estes tratamentos requerem altas concentrações de produtos por um longo período, desencadeando elevados custos e impactos ambientais, bem como o risco de aumentar a resistência do parasito a estes recursos (PICÓN-CAMACHO *et al*., 2012). Portanto, os tratamentos mencionados são limitados pela toxicidade, rentabilidade e difícil controle em pisciculturas de grande porte (SUTILI *et al*., 2014). Em vista deste problema, um estudo abordou a modificação do pH da água e dureza, sendo então constado que essa infecção é menos grave em *R. quelen* quando a água for mantida em pH 5 e dureza igual a 20 mg $.L^{-1}$ de $CaCO_3$ (GARCIA *et al*., 2011).

2.4 *Rhamdia quelen*

A espécie *Rhamdia quelen* (Figura 3) pertence à família Heptapteridae e é popularmente conhecida jundiá, jundiá-tinga, jandiá, jandiá-tinga, mandi, sapipoca, bagre, bagre negro, bagre-sapo, bagre sulamericano, nhurundia e mandi-Guaru. O jundiá tem coloração que varia de marrom avermelhado claro a cinza ardósia e possui característica ectodérmica e tem ampla distribuição geográfica, sendo encontrado desde o sudeste do México até o sul da Argentina. Habita preferencialmente fundos arenosos de lagos e rios de águas calmas, possuindo hábitos noturnos, alimentação omnívora, com tendência piscívora e podendo atingir cerca de 50 cm de comprimento e 3Kg de peso corporal (GOMES *et al*., 2000).

O jundiá é a espécie nativa mais cultivada no RS, uma vez que é tolerante às baixas temperaturas do inverno. Ainda, apresenta facilidade de adaptação em âmbito laboratorial e vem sendo utilizada há anos pelo nosso grupo de pesquisas. Os diversos trabalhos têm como objetivo testar modelos experimentais que envolvem pesquisa para melhoramento de dieta, avaliação de fatores de crescimento, reprodução, prevenção/tratamento de patologias que acometem peixes de água doce, desenvolvimento de sedativos e anestésicos para uso em

piscicultura, entre outros (SUTILI *et al*., 2014; GOMES *et al*., 2000; GARCIA *et al*., 2011 SILVA *et al*., 2013; LAZZARI *et al*., 2011; BARCELLOS *et al*., 2014).

Figura 3 – Foto de um exemplar de *Rhamdia quelen*

Fonte: Internet

Os peixes teleósteos são os primeiros da classe de vertebrados com imunidade inata e adaptativa (WHYTE, 2007). Estas respostas são moduladas por fatores intrínsecos e extrínsecos, como a idade, temperatura, contato com herbicidas e alimentação, entre outros fatores. Nesses animais, os macrófagos teciduais são as principais células do sistema imune inato e representam uma das primeiras e mais importantes barreiras frente à micro-organismos patogênicos. Também têm a capacidade de fornecer sinalização para os linfócitos, que atuam na imunidade adquirida (GUEDES, 1980; SILFVERGRIP, 1996; FRACALOSSI *et al*., 2002; KREUTZ *et al*., 2010; AOKI *et al*., 2013). Entretanto, o jundiá possui susceptibilidade frente ao protozoário conhecido como "ich" (*Ichthyophthirius multifiliis*), sendo que o cultivo desta espécie em sistemas de aquicultura, como tanques e aquários, por vezes pode desencadear uma contaminação em massa por agentes infectantes ou até mesmo uma co-infecção por parasitos ou bactérias, ocasionando uma perda considerável na produção (SUTILI *et al*., 2013).

A espécie *Rhamdia quelen* (jundiá) não possui seus parâmetros hematológicos e bioquímicos bem elucidados, pois de acordo com a literatura esses parâmetros são bastante variáveis e ainda não possuem valores normais e intervalos de confiança bem estabelecidos. Ainda embasando-se na literatura, tem-se que através do sangue podem ser evidenciadas várias alterações metabólicas. Também pode ser avaliado o estado de saúde dos animais e eventuais enfermidades nos mesmos, além de contribuir para a compreensão da fisiologia,

relação filogenética interespécies e outros parâmetros ecológicos provenientes da intervenção de produtos comerciais/ naturais (TAVARES-DIAS *et al*., 2000; LAZZARI *et al*., 2011; BARCELLOS *et al*., 2014).

3 MANUSCRITO

The essential oil *Hyptis mutabilis* against *Ichthyophthirius multifiliis* and its effect on hematological, biochemical and immunological parameters in silver catfish (*Rhamdia quelen*)

Cunha, J.A.; Sutili, F.J.; Motta, A.O.; Gressler, L.T.; Scheeren, C.A; Silva, L.L.; Almeida, R.V.; Baldisserotto, B.; Heinzmann, B.M

Artigo submetido ao periódico Veterinary Parasitology

The essential oil *Hyptis mutabilis* against *Ichthyophthirius multifiliis* and its effect on hematological, biochemical and immunological parameters in silver catfish (*Rhamdia quelen*)

Cunha, J.A[1].; Sutili, F.J[1].; Motta, A.O[2].; Gressler, L.T[3].; Scheeren, C.A[2]; Silva, L.L[4].; Almeida, R.V[5].; Baldisserotto, B[1,6].; Heinzmann, B.M[1,2,7].

[1]Programa de Pós-Graduação / PPG em Farmacologia, Federal University of Santa Maria / UFSM, Campus Universitário, Prédio 21, BR-97105-900, Santa Maria, RS, Brazil.

[2] Curso de Graduação em Farmácia, UFSM, Santa Maria, RS, Brazil.

[3] PPG em Medicina Veterinária, UFSM, Santa Maria, RS, Brazil.

[4] Universidade Regional Integrada do Alto Uruguai e das Missões/ URI, Santiago, RS, Brazil.
[5] PPG em Análises Clínicas, Centro Universitário Franciscano, Santa Maria, RS, Brazil.
[6] Departamento de Fisiologia e Farmacologia, UFSM, Santa Maria, RS, Brazil.
[7] Departamento de Farmácia Industrial, UFSM, Santa Maria, RS, Brazil.

Phone: (55) 32209674; email: berta.heinzmann@gmail.com; Address: UFSM/ CCS /DFI, Prédio 26, Campus Universitário, BR-97105-900, Santa Maria, RS, Brazil.

ABSTRACT

The aim of this study was to evaluate the activity of leaves essential oil (EO) of *Hyptis mutabilis*, as well as its major constituent (-)-globulol against the fish parasite *Ichthyophthirius multifiliis* (Ich) and their effects on hematological, biochemical and immunological parameters in silver catfish (*Rhamdia quelen*). In the first experiment lasting four days, naturally infected fish were treated with EO (0, 10 and 20 mg.L^{-1} and 200 µL.L^{-1} ethanol) by means of different exposure methods (a single application at the beginning of the experiment, twice with a 48 h interval, and 1 h daily baths). Fish mortality and the number of parasites (trophonts per fish) were assessed after 48 and 96 h. The 1 h daily baths provided the best survival when compared to a single application and two applications with a 48 h interval, and this methodology was set for the second experiment, in which infected animals were exposed to (-)-globulol at 2.5 and 5 mg.L^{-1}. The most effective concentrations in the experiments 1 and 2 were chosen for experiment 3, in which healthy animals were subjected to 1 h daily baths with EO (20 mg.L^{-1}) or (-)-globulol (2.5 mg.L^{-1}). Four days later, the

animals had hematological, biochemical, and immunological parameters assessed. After exposure of healthy animals to EO, significant increase was detected in hematocrit (28.42 %), erythrocytes (2.11 x 10^6 μL^{-1}) and leukocytes (48.82 x 10^3 μL^{-1}), while the total count of thrombocytes (20.50 x 10^3 μL^{-1}) decreased in comparison to previous studies with *Rhamdia quelen*. However exposure to (-)-globulol increased only the leukocyte number (70.87 x 10^3 μl^{-1}) related to the default values already reported to this species. Total cholesterol showed elevated levels (226.66 $mg.dL^{-1}$) in fish exposed to EO, while the group treated with (-)-globulol presented higher LDL (244.66 $mg.dL^{-1}$) and lower HDL (87.66 $mg.dL^{-1}$) values than the control and ethanol groups. EO and (-)-globulol increased survival of fish infected with ich and altered some hematological and biochemical parameters, such as such as albumin, total cholesterol, HDL, LDL, hematocrit, erythrocytes, leucocytes, thrombocytes and MCV. No significant differences were detected in nonspecific immune assay between the treated groups and control, indicating that the EO and (-)-globulol did not increase innate immunity of fish.

Key-words: organic aquaculture, biological control, (-)-globulol, parasitic disease

1. INTRODUCTION

The silver catfish (*Rhamdia quelen*) is the most raised native species from southern Brazil (Baldisserotto *et al.*, 2009) because presents good food conversion, growth rate and absence of intramuscular spines (Gomes *et al*., 2000). However, this species is very susceptible to the protozoan parasite *Ichthyophthirius multifiliis* (ich) (Miron *et al*., 2003; Garcia *et al*., 2007). Farming of silver catfish in aquaculture systems such as ponds and aquaria sometimes may trigger a massive contamination by infectious agents or even a co-infection with bacteria (*A. hydrophila*) or parasites (*I. multifiliis*) (Sutili *et al*., 2013). The infected animals present white spots and characteristic behavior, which includes swimming imbalance and injuries caused when the fish scratch the tank walls as a result of irritation, and can also lead to respiratory distress, severe agitation, loss of appetite and death as a result of susceptibility to secondary infections (Matthews, 2005; Garcia *et al*., 2007; Sutili *et al*., 2013).

Some treatments were used against ich, such as the addition of salt (Miron *et al*., 2003), copper sulfate (Straus, 2008; Straus *et al*., 2009), potassium permanganate (Straus & Griffin, 2002) and increasing temperature. However, these treatments are limited by toxicity

and/or low profitability and control in large fish farms (Carneiro *et al*., 2005; Picón-Camacho *et al*., 2012). In this context, an alternative increasingly considered are essential oils (EO), complex mixtures of volatile compounds from plants that are a source of pharmacologically active constituents and may trigger analgesic, anti-inflammatory, antibiotic and repellant effects, among others (Curtis *et al*., 1989; Gillij *et al*., 2007; Nerio *et al*., 2010).

Plants from the Lamiaceae family are known to be sources of EO, among which stands out *Hyptis mutabilis* (Rich.) Briq., used in folk medicine due to its antimicrobial, larvicide, sedative, anti-inflammatory and antinociceptive effects (Fachinetto,Tedesco, 2009; Ximenes *et al*., 2013; Silva *et al*., 2013). The EO of this species collected in southern Brazil has in its composition mono- and sesquiterpenoids and as the major constituent (-)-globulol (26.61%) when the EO is derived from leaves, and Germacrene D (14.97%) when from inflorescences (Silva *et al*., 2013). This study aimed to evaluate the *in vivo* antiparasitic activity of the EO of *Hyptis mutabilis* leaves and of (-)-globulol against ich using different methodologies. Hematological, biochemical and immunological parameters of healthy animals were also evaluated, after treatment by the methodology that promoted the highest survival of parasitized animals during the tests.

2. MATERIALS AND METHODS

2.1. Plant material and phytochemical analysis

Leaves of *Hyptis mutabilis* were collected in March 2012 (at the end of summer) in Santa Maria (Rio Grande do Sul state, southern Brazil). A voucher specimen was deposited at the Herbarium of the Departamento de Biologia, Universidade Federal de Santa Maria (N: SMDB 13076). The EO was extracted and analyzed as described by Silva *et al*. (2013), which also reported the isolation of (-)-globulol from the EO by column chromatography, identification of this constituent by EI-MS, ^{1}H- and ^{13}C-NMR. The measure of optical rotation by these authors defined the constituent as the levorotatory isomer.

2.2. Fish and water quality

Silver catfish (*R. quelen*) juveniles (Exp 1: 5.3 ± 0.40 g, Exp 2: 25 ± 0.50 g and Exp 3: 5.6 ± 0.30 g) were used to evaluate survival and immunological, biochemical and hematological parameters after exposure. All fish were transferred from a local fish culture to the laboratory, where they were maintained in continuously aerated 250 L tanks, with controlled water parameters until experiments were performed. The acclimation tanks had

70% of water exchanged each 48 h. Dissolved oxygen and temperature were measured with an YSI oxygen meter (Model Y5512) and the pH by a DMPH-2 pH meter (Digimed). Total ammonia levels were determined according to Verdouw *et al*. (1978) and un-ionized ammonia (NH3) levels were calculated according to Ostrensky (1997). Fish were acclimated for seven days and were fed once a day with commercial feed (28.0% crude protein). Juveniles were fasted for a period of 24 h prior to the experiments.

The mean water quality parameters in experiment 1 were: temperature: 21.0 ± 2 °C, pH: 6.8 ± 0.11, dissolved oxygen levels: 7.98 ± 0.12 $mg.L^{-1}$, total ammonia levels: 2.08 ± 0.21 $mg.L^{-1}$, non-ionized ammonia: 0.0279 ± 0.0008 $mg.L^{-1}$; experiment 2 were: temperature: 21.0 ± 2 °C, pH: 7.1 ± 0.08, dissolved oxygen levels: 5.8 ± 1.65 $mg.L^{-1}$, total ammonia levels: 1.20 ± 0.15 $mg.L^{-1}$, non-ionized ammonia: 0.0279 ± 0.0006 $mg.L^{-1}$; and in experiment 3 were: temperature: 21.0 ± 2 °C, pH: 7.3 ± 0.05, dissolved oxygen levels: 7.2 ± 1.15 $mg.L^{-1}$, total ammonia levels: 1.80 ± 0.3 $mg.L^{-1}$, non-ionized ammonia: 0.0242 ± 0.0007 $mg.L^{-1}$.

2.3. Experiment 1

All experiments were conducted with water (control) and ethanol (at the same concentration used for dilution of the highest concentration of EO). The EO was diluted in 95% ethanol (1:10) and added separately to 1.5 L aquaria, while (-)-globulol was added directly to the water. Fish infected with *I. multifiliis* (in quadruplicate with n = 5 fish for each replicate and about 20 white spots per fish) were exposed for four days to EO at 0, 10 and 20 $mg.L^{-1}$ and 200 $\mu L.L^{-1}$ ethanol using three different methodologies: a single application at the beginning of the experiment (A), twice with a 48 h interval (B) and 1 h daily baths (C). To evaluate the antiparasitic activity of EO, lower concentrations than those reported as sedative were chosen (Silva *et al*., 2013). Parasites were counted in the dorsolateral region above the lateral line with a magnifying glass (x 10) at the beginning of the experiment, after 48 h, and at the end of the experiment (96 h). Daily mortality in each group was also assessed through the same period.

2.4. Experiment 2

Silver catfish infected with ich (in quadruplicate, with n = 5 fish for each replicate and about 18 white spots per fish) were exposed for four days to (-)-globulol at 2.5 and 5 $mg.L^{-1}$ through 1 h daily baths. The globulol concentrations chosen for the experiment 2 were lower

than those which showed sedative effect in silver catfish (Silva *et al*., 2013). The counting of parasites and the assessment of mortality were performed as described in experiment 1.

2.5. Experiment 3

According to the results of the first and second experiments (survival experiments), the optimum exposure method and the most effective concentrations of EO and (-)-globulol were chosen in order to investigate possible effects on fish metabolism. Thus, healthy animals were subjected to 1 h daily baths for four days at 20 $mg.L^{-1}$ EO or 2.5 $mg.L^{-1}$ (-)-globulol. The methodology of all these experiments was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process N: 046/2010).

2.6. Blood collection-Experiment 3

At the end of the fourth day of the experiment 3, fish from each aquarium were anesthetized with eugenol (50 $mg.L^{-1}$), blood was collected with heparinized syringes and stored in eppendorf tubes for later hematological analysis. The material directed to biochemical and immunological analysis was stored in another eppendorf tubes, which were centrifuged (1500 x g, room temperature,1 min) to separate serum and plasma.

2.7. Hematological analysis

Hematological parameters were evaluated at the end of experiment 3, using an automatic counter XS-800i (Symex®). Total leukocytes, total erythrocytes, hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and platelets were determined. Blood smears were fixed in methanol and stained with Instant-Prov (NewProv®) for determination of the differential white blood cell (WBC) count. At least 200 WBCs were counted for differential WBC determinations.

2.8. Serum biochemistry

The serum levels of glucose, total proteins, albumin, total bilirubin, creatinine, urea, total cholesterol, triglycerides, HDL and LDL cholesterol were evaluated at the end of experiment 3 in an automated Vitros 250 (Ortho – Clinical Diagnostics) using Johnson & Johnson kits dry chemistry method. All tests were carried out in duplicate.

2.9. Non-specific immune assays

The complement system activity was determined at the end of experiment 3, according to Castro *et al.* (2008) using fresh plasma (without freezing) from each fish. Rabbit red blood cells were added to a normal plasma and incubated at 20 ºC, after 1 h ice-cold saline was added to stop the complement activity, cells were pelleted by centrifugation and the absorbance of supernatant was measured in a microplate reader (405 nm). The percent hemolysis was calculated by comparison between total hemolysis (100%) and no-hemolysis (0%) controls.

The natural agglutination activity of fish plasma was investigated using "U"-shaped 96-well plates. Plasma was diluted two-fold in PBS (pH 7.4, Ca^{+2} and Mg), and an equal volume of washed *Aeromonas hydrophila* (ATCC 7966) in suspension (0.4 OD 600 nm) was added to each well. The plates were incubated for 2 h at 25 ºC and then overnight at 4 ºC; the titer was defined as the logarithm of the highest dilution of the plasma that caused complete agglutination of the bacterial cell (Sutili *et al*., 2014).

Plasma bactericidal activity was assessed against *A. hydrophila* (ATCC 7966) by agar diffusion method, using paper discs (5 mm diameter). The bacteria (McFarland $0.5x10^8$ cells.mL^{-1}) were seeded in Petri dishes (solid medium Müller Hinton) and plasma was soaked on the discs (20 µL). The plates were incubated at 30 ºC for 24 h, and afterwards the diameter of the halo formed around the discs was assessed (Sutili *et al*., 2014).

2.10. Statistical analysis

The homogeneity of variances between groups was determined by Levene test. Comparisons between different groups were performed using one-way ANOVA and Tukey's test (Statistica 7.0 Software). Fish survival was compared using Kaplan-Meier survival analysis with the Log-rank test (SPSS 18 Software). The minimum significance level was set at $P \leq 0.05$. Hematological, biochemical and immunological parameters are presented as mean values and standard error.

3. RESULTS

3.1. Effect of EO of *H. mutabilis* and (-)-globulol against *Ichthyophthirius multifiliis* and fish survival

Experiment 1 - control and ethanol groups showed survival of 60% and 40% after 96 h of exposure, respectively. The group treated with a single exposure to 10 $mg.L^{-1}$ EO presented equivalent survival as the control, but fish submitted to 20 $mg.L^{-1}$ EO had significantly higher

survival ($P \leq 0.01$) (100%) than the control (Fig 1A). The control group of the two applications methodology showed 20% survival at the end of 96 h of exposure. Fish treated with EO and ethanol showed higher survival compared to the control ($P \leq 0.01$) (Fig. 1B). The survival of the control and ethanol groups were 50 and 80%, respectively, after 96 h exposure with daily baths. Both groups treated with EO presented higher survival ($P \leq 0.01$) than the control and ethanol group (Fig. 1C).

The number of trophonts per fish was calculated considering the average values (n=20) for the intervals of 0, 48 and 96 hours, for each of the treatments. In methodology A, ethanol group showed averages of 8.2, 10 and 75 at the above mentioned times, while for the control group, the corresponding averages were 8.4, 11.2 and 53.3. At the concentration of 10 $mg.L^{-1}$ EO, averages of 6.3, 5.4 and 17.3 were found, and averages of 7.9, 8.2 and 39.2 were observed for the concentration of 20 $mg.L^{-1}$ EO. However, for methodology B, the ethanol group had means of 8.7, 5, 47.33, respectively, while for the control averages of 9.6, 1.25 and 1 were observed. The averages detected at the concentration of 10 $mg.L^{-1}$ EO were 8.7, 13.9 and 35.3 and at 20 mg L^{-1} EO the corresponding values were 9.1, 5.3 and 41.66. In the methodology C, the ethanol group showed values of 15.8, 24.33 and 11.5, while for the control 9.3, 3.5 and 11 were observed. However, at the concentration of 10 $mg.L^{-1}$ EO, 13.8, 48.2 and 20.2 were found, while at 20 $mg.L^{-1}$ EO, 13.1, 24.66 and 19.8 were observed (Fig. 2A-C).

When a single application of EO was used, the control group differed from ethanol, 10 and 20 mg.L-1 EO after 48h; at the same time, 10 $mg.L^{-1}$ EO was different from ethanol, control and 20 $mg.L^{-1}$. However, after 96 h, control group and 10 $mg.L^{-1}$ EO differed from ethanol, while 20 $mg.L^{-1}$ EO differed from control and 10 $mg.L^{-1}$ EO (Fig. 2A). The treatment that received two applications within 48 h, after the first 48 h 10 $mg.L^{-1}$ EO differed from ethanol, control group and 20 $mg.L^{-1}$ EO (Fig. 2B). The 1 h daily baths treatment showed statistic difference between 10 $mg.L^{-1}$ EO and ethanol after 48 h (Fig. 2C).

Statistical differences were also observed for the same methodology at different times. In methodology A, differences were observed among the control, ethanol and 20 $mg.L^{-1}$ EO after 96 h when compared to the other two evaluated times (Fig. 2A). In the methodology B, no difference was detected after 96 h when compared to the other two times evaluated in ethanol group, 10 and 20 $mg.L^{-1}$ EO. There was also no difference in the control group between the times 0 and 48 h (Fig. 2B). In methodology C, the group subjected to a concentration of 10 $mg.L^{-1}$ EO showed statistic difference among different times (Fig. 2C).

Experiment 2 - After 96 h from the beginning of the experiment, the survival observed for fish submitted to 1 h daily baths of (-)-globulol 2.5 mg.L^{-1} was significantly highest ($P \leq 0.01$) than the other treatments (Fig. 1D). The fishes which were exposed to (-)-globulol at 2.5 and 5 mg.L^{-1} through 1 h daily baths (Fig. 1D) revealed a difference between 5 mg.L^{-1} and ethanol, while water and 2.5 mg.L^{-1} showed to be different after 48 hours. For 96 hours interval, (-)-globulol 5 mg.L^{-1} differed from ethanol and control group; ethanol and control group were different from all other treatments, including each other.

Concerning the number of trophonts per fish, the average numbers after 0, 48 and 96 h observed for the ethanol group were 18.7, 57 and 5.3, whereas for the control group the corresponding data were 15.4, 51.2 and 71.3. For 2.5 mg.L^{-1} (-)-globulol, averages of 16.3, 73.4 and 50.3 were obtained, while for 5 mg.L^{-1} (-)-globulol, 20, 37.2 and 39.2 were found.

Considering the three evaluated time periods, a significant statistic difference were observed for the control group and 2.5 mg.L^{-1} (-)-globulol at time 0 h when compared to 48 and 96 h; at 0 and 96 h ethanol group was different when compared to 48 h (Fig. 2D).

Significant difference between treatments after 48 h was detected among the group subjected to 5 mg.L^{-1} (-)-globulol in relation to the control, ethanol and 2.5 mg.L^{-1} (-)-globulol groups within the same time. After 96 h, there was a difference of 5 mg L^{-1} (-)-globulol when compared to the control and ethanol groups, and at this same time the ethanol group differed from all other treatments (Fig 2D).

Experiment 3 – Daily baths of EO (20 mg.L^{-1}) and (-)-globulol (2.5 mg.L^{-1}) with respect to infected fish survival showed no significant difference between the two treatments, however both groups presented higher survival than the control and ethanol groups (Fig. 1E).

3.2. Effect of essential oil of *H. mutabilis* and (-)-globulol on hematological and biochemical parameters and non-specific immune assays

Of the ten biochemical parameters evaluated in serum, HDL presented significantly lower values in fish subjected to baths with 2.5 mg.L^{-1} (-)-globulol compared to control fish. Albumin showed high values for ethanol compared to the control group. The LDL parameter presented lower values for the control group and ethanol when compared with 20 mg.L^{-1} EO. For total cholesterol, higher values were obtained for the animals treated with 20 mgL^{-1} EO in comparison to other treatments with ethanol and 2.5 mg.L^{-1} (-)-globulol, and to the control group (Table 1).

Considering the analyzed hematological parameters, hematocrit showed higher levels in fish exposed to EO (20 $mg.L^{-1}$) compared to control group. The treatment with (-)-globulol (2.5 $mg.L^{-1}$) showed a significant increase in the total number of erythrocytes and leukocytes when compared to control group. The number of thrombocytes were lower in fish exposed to 20 $mg.L^{-1}$ EO compared to control, ethanol and groups, (-)-globulol (2.5 $mg.L^{-1}$). However, MCV values were higher in the control group in comparison to 2.5 $mg.L^{-1}$ (-)-globulol and 20 $mg.L^{-1}$ EO (Table 2).

The complement system activity represented by hemolysis of fish erythrocytes showed no significant differences for all treated groups compared to control (Table 2). Titer and halo formation in the plasma agglutination and plasma bactericidal assays were not verified in non-specific immune assays avaliable.

4. DISCUSSION

Ichthyophthirius multifiliis has the ability to infect all freshwater fish species and is considered an important research field due to the significant economic impact on fish farming. It is also a good model for the study of innate and acquired immunity against fish parasites in teleost (Dickerson, Clark, 1998; Dickerson, 2012; Matthews, 2005).

In the present study, a decrease of the number of white spots could not be observed. However animals treated with EO or (-)-globulol showed a higher survival compared to untreated fish. Effective alternatives to combat the great losses caused by ich are the use of 4 g. L^{-1} of common salt (Miron *et al*., 2003) or 1.1 $mg.L^{-1}$ $CuSO_4$ (Straus, 2008). Salt prevents the fish deaths and progressively reduces the number of trophonts, however its use is not feasible in large aquaculture systems. Although $CuSO_4$ has showed efficacy in a study (Straus, 2008) it also presented high toxicity (Straus, Griffin, 2002; Straus *et al*., 2009).

Research with EO have focused on this problem, since according to the Food and Agriculture Organization the use of plant extractives as EO are allowed to control parasites in organic fish farming (Prein *et al*., 2012). Reports of *in vitro* activity of *Melaleuca alternifolia*, *Lavandula angustifolia* and *Mentha piperita* EO purchased commercially against *I. multifiliis* have been found, and the EO of *M. alternifolia* was effective to treat ichthyophthiriasis in pacu (*Piaractus mesopotamicus*) (Valladão, 2014).

The blood may show various metabolic changes and also provides an animal health condition assessment and detection of any diseases. These parameters can also contribute to understand the physiology, interspecies phylogenetic relationship and other ecological

parameters from intervention commercial/ natural products (Tavares-Dias *et al*., 2000; Lazzari *et al*., 2011; Barcellos *et al*., 2014). In this study the control group values of serum glucose, albumin, total proteins, total bilirubin, total cholesterol, creatinine and urea were similar to basal values reported for the species (Borges *et al*., 2004). However, for triglycerides and LDL levels were lower, while HDL was higher when compared to those already reported (Borges *et al*., 2004). There are no literature data addressing a possible explanation for the increase in LDL and HDL observed in *R. quelen*. The obtained results for the hematological parameters erythrocytes, hemoglobin, hematocrit, MCHC, MCV, heterophils and eosinophils were similar to those described by Tavares-Dias, *et al*. (2000). Regarding to total thrombocyte number, a decrease of this parameter in animals exposed to EO and also an increase of total leucocyte number in fish treated with EO and globulol were observed. The thrombocytes represent an important linkage between innate and adaptive immunity in fish, which interaction is involved in protection against pathogenic and environmental factors (Passantino *et al*., 2005). The role of thrombocytes in fish defense mechanisms has been quite studied but is still controversial because there is no evidence of phagocytosis by thrombocytes (Meseguer *et al*., 2002). Leukocytes are responsible for defending the organism against infections and parasites, acting in the production of antibodies (Misra *et al*., 2006; Ranzani-Paiva *et al*., 2004). Although the intervention with the OE and globulol was not statistically different from the control in the immunoassay, hematology results corroborate with the increased survival.

A wide range of EO from aromatic medicinal plants consists of many active substances that are known to trigger inhibitor or stimulating effects on the immune system and may also have immunomodulatory properties in fish (Sutilli *et al.*, 2014). However, in this study there was no significant change in the immunological test to determine the hemolysis percentage of fish erythrocytes of treated fish when compared to control. Fish immune system is classified into innate or nonspecific defense, which is the first line of protection against pathogens, and specific defense, characterized by the immune memory (Biller-Takahashi *et al*., 2012). The complement system activity is widely used as an immune indicator (Bayne, Gerwick, 2001), and it was evaluated to measure the hemolytic activity of the alternative pathway from complement system, as an indicator of the innate immunity in silver catfish after being and submitted to 1 h daily baths with EO or (-)-globulol. Despite the increased survival of infected animals, hemolysis of treated fish erythrocytes showed no significant difference from control. In addition, the significant differences detected in the

number of trophonts per fish in relation to the control group could not be assigned to an antiparasitic activity, since the reduction in the number of trophonts was directly related to mortality of the specimens under study. However, sedative and anesthetic activities detected for *H. mutabilis* EO and (-)-globulol may be contributing to the increased survival of infected animals. Since (-)-globulol showed positive interaction with diazepam regarding the depressor effects, (Silva *et al*., 2013), its sedative effect may be preventing the decline of the immune defenses resulting from stress.

It is also noteworthy that factors such as nutritional status, seasonality, maturation, sex and genetic variation can also significantly influence the hematological variables. Furthermore, differences may occur in the blood collection methodology, regarding the type of anticoagulant used, which can also act as a source of variation in fish hematology results (Tavares-Dias *et al*., 2000). Thus, a clear explanation for the obtained results is still missing. However, the lack of significant difference in the survival increase of infected fish treated with EO (20 $mg.L^{-1}$) or with globulol (2.5 $mg.L^{-1}$) allows us to suggest that the tested products can be an alternative for the control of parasites in both organic as conventional aquacultures.

5. CONCLUSION

Daily baths of the EO of *H. mutabilis* (20 $mg.L^{-1}$) and its major constituent (-)-globulol (2.5 $mg.L^{-1}$) increased the survival rate of silver catfish infected with ich. The number of trophonts per fish changed over time and among treatments. However, the detected differences could be attributed to mortality of animals, excluding a possible antiparasitic activity of EO and its major constituent at the concentrations used in this study. Changes in some hematological and biochemical parameters were observed in healthy fish, but no clear relationship between them and higher survival of infected fish was found.

6. ACKNOWLEDGMENTS

This study was supported by CNPq (#454447/2014-0), Ministry of Fisheries and Aquaculture/FINEP (#01.12.0130.00) and ADAPTA/FAPEAM/CNPq (#573976/2008-2). B. Baldisserotto is grateful to CNPq for research fellowship; Cunha, J. A., Sutili, F. J., and Gressler, L. T. are grateful to CAPES for their graduate fellowships. Motta, A. O. and Scheeren, C. A. are grateful to FIT/UFSM for their undergraduate fellowships.

7. REFERENCES

Baldisserotto, B., 2009. Fisiologia de peixes aplicada à piscicultura. 2. ed. Santa Maria: Universidade Federal de Santa Maria.

Barcellos, L. J. G. *et al.* 2014. Hematological changes in jundiá (*Rhamdia quelen* Quoy and Gaimard Pimelodidae) after acute and chronic stress caused by usual aquacultural management, with emphasis on immunosuppressive effects. Aquaculture, 237, 229–236.

Bayne, C.J., Gerwick, L., 2001. The acute phase response and innate immunity of fish. Dev. Comp. Immunol. 25, 8, 725-743.

Biller-Takahashi, J. D., Takahashi, l. S., Marzocchi-Machado, C. M., Zanuzzo, F. S., Sabioni, R. E., Urbinati, E. C., 2012. Hemolytic activity of alternative complement pathway as an indicator of innate immunity in pacu (*Piaractus mesopotamicus*). Rev. Bras. Zootec. – Braz. J. Anim. Sci. 41, 2, 237-241.

Borges, A., Scotti, L.V., Siqueira, D.R., Jurinitz, D.F., Wassermann, G.F., 2004. Hematologic and serum biochemical values for jundiá (*Rhamdia quelen*). Fish Physiol. Biochem. 30, 21–25.

Castro, S.B.R., Leal, C.A.G., Freire, F.R., Carvalho, D.A., Oliveira, D.F., Figueiredo, H.C.P., 2008. Antibacterial activity of plant extracts from Brazil against fish pathogenic bacteria. Braz. J. Microbiol. 39, 756-760.

Carneiro, P.C.F., Schorer, M., Mikos, J.D., 2005. Tratamentos Terapêuticos convencionais no controle do ectoparasita *Ichthyophtirius multifiliis* em jundiá (*Rhamdia quelen*). Pesqu. Agropecu. Bra. 40, 99–102.

Curtis, C., Lines, J., Lu, B., Renz, A., 1989. Natural and synthetic repellents. In: Curtis, C.F. Editor. Appropriate Technology in Vector Control. CRC Press, Florida, pp.76–89.

Dickerson, H., Clark, T., 1998. *Ichthyophthirius multifiliis*: a model of cutaneous. Immunol. Rev. 166, 377-84.

Dickerson, H. W., 2006. *Ichthyophthirius multifiliis* and *Cryptocary onirritans* (*phylum Ciliophora*). In: Woo, P.T.K. (Ed.), Fish Diseases and Disorders, 1. Protozoanand Metazoan Infections, 2nd ed. CAB International, Wallingford, UK, pp. 116–153.

Dickerson, H. W., 2012. *Ichthyophthirius multifiliis*. In: Patrick, K. B., Woo, T. K. (Eds.), Fish Parasites Pathobiology and Protection. CABI, Wallingford, pp. 55–72.

European Directorate for the Quality of Medicines, 2007. European Pharmacopeia. 6th edn. Strassbourg.

Fachinetto, J. M. Tedesco, S. B., 2009. Número cromossômico, análise meiótica e estimativa da viabilidade polínica em populações de *Hyptis mutabilis* (Rich.) Briq. Rev. Bras. Plantas Med. 11, 1, 110-116.

Garcia, L. O., Becker, A. G., Copatti, C. E., Radunzneto, J., Baldisserotto, B. 2007. Salt in the food and water as a supportive therapy for *Ichthyophthirius multifiliis* infestation on silver catfish, *Rhamdia quelen*, fingerlings. J. World Aquacult. Soc. 38, 1–11.

Gillij, G. Y., Gleiser, R. M., Zygadlo, J. A. 2007. Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. Bioresour. Technol. 99, 2507–2515.

Gomes, L.C., Golombieski, J.I., Chippari-Gomes, A.R., Baldisserotto, B., 2000. Biologia do jundiá *Rhamdia quelen* (TELEOSTEI, PIMELODIDAE). Ciênc. Rural, 30, 179-185.

Lazzari, C.R. *et al.* 2011. Hematologia de júndias em resposta ao nível de proteína. Ciênc. Anim. Bras. 12, 2, 192-197.

Matthews, R. A., 2005. *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriosis in freshwater teleosts. Adv. Parasitol. 59, 159–241.

Meseguer, J., Angeles Esteban, M., Rodríguez, A., 2002. Are thrombocytes and platelets true phagocytes. Microsc. Res. Techniq. 57, 491-497

Miron, D. S., Silva, L. V. F., Golombieski, J.I., Baldisserotto, B. 2003. Efficacy of different salt (NaCl) concentrations in the treatment of *Ichthyophthirius multifiliis* contamination of silver catfish, *Rhamdia quelen*, fingerlings. J. Appl. Aquac. 14, 155- 161.

Misra, C.K., Das, B.K.., Mukherjee, S.C., Meher, P.K., 2006. The immunomodulatory effects of tuftsin on the non-specific immune system of Indian Major carp, *Labeo rohita*. Fish Shellfish Immunol. 20, 728-738.

Nerio, L. S., Jesus, O. V., Stashenko, E. 2010. Repellent activity of essential oils: A review. Bioresour. Technol. 101, 372–378.

Ostrensky, A. 1997. Estudos para viabilização tecnológica dos cultivos de camarões marinhos no litoral do Estado do Paraná, Brasil. Tese de doutorado. Universidade Federal do Paraná, Curitiba, PR. Brasil. 126.

Passantino, L., Cianciotta, A., Patruno, R., Ribaud, M.R., Jirillo, E., Passantino, G.F., 2005. Do fish thombocytes play an immunological role? Their cytoenzimatic profiles and fuction during an accidental piscine candidiasis in aquarium. Immunopharmacol. Immunotoxicol. 27, 345-356.

Picón-Camacho, S. M. P. M., Marcos-Lopez, M., Bron, J. E., Shinn, A. P., 2012. An assessment of the use of drug and non-drug interventions in the treatment of *Ichthyophthirius multifiliis* Fouquet, 1876, a protozoan parasite of fresh water fish. Parasitology, 139, 149–190.

Prein, M., Bergleiter, S., Ballauf, M., Brister, D., Halwart, M., Hongrat, K., Kahle, J., Lasner, T., Lem, A., Lev, O., Morrison, C., Shehadeh, Z., Stamer, A.,Wainberg, A.A., 2012. Organic aquaculture: the future of expanding niche markets. FAO, Rome and NACA, Bangkok. Avaliable from: http://www.fao.org/docrep/015/i2734e/i2734e04c.pdf. Accessed on 27.01.2015.

Ranzani-Paiva, M.J.T., Ishikawa, C.M., Eiras, A.C., Silveira, V.R., 2004. Effects of an experimental challenge with mycobacterium marinum on the blood parameters of nile tilapia, *Oreochromis niloticus* (Linnaeus, 1757). Braz. Arch. Biol. Technol. 47, 6, 945-953.

Silva L. L., *et al*. 2013. Sedative and anesthetic activities of the essential oils of *Hyptis mutabilis* (Rich.) Briq. and their isolated components in silver catfish (*Rhamdia quelen*). Braz. J. Med. Biol. Res. 46, 771-779.

Straus, D.L., 2008. Comparison of copper sulfate concentrations to control ichthyophthiriasisin fingerling channel catfish. J. Appl. Aquac. 20, 4, 272-284.

Straus, D.L. Griffin, B.R., 2002. Efficacy of potassium permanganate in treating ichthyophthiriasis in channel catfish. J. Aquat. Anim. Health, 14, 2, 145-148.

Straus D.L., Hossain, M.M., Clark, T.G., 2009. Copper sulfate toxicity to two isolates *Ofichthyophthirius multifiliis* relative to alkalinity. Dis. Aquat. Org. 83, 1, 31-36.

Sutili, F. J., Gressler, L. T., Vargas, A. P. C., Baldisserotto, B., Cunha, M. A. 2013. The use of nitazoxanide against the pathogens *Ichthyophthirius multiliis* and *Aeromonas hydrophila* in silver catfish (*Rhamdia quelen*). Vet. Parasitol. (Print), 522 - 526.

Sutilli, F. J., *et al*. 2014. The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*). Vet. Immunol. Immunopathol. 157, 142–148.

Tavares-Dias, M., Schalch, S. H. C., Martins, M. L., *et al*. 2000. Características hematológicas de *Oreochromisn iloticus* (Osteichthyes: Cichlidae) cultivadas intensivamente em "Pesque-Pague" do Município de Franca, São Paulo, Brasil. Ars Vet. Jaboticabal, 16, 2, 76-82.

Valladão, G. M. R., 2014. Potencial de óleos essenciais de plantas para o tratamento de enfermidade em peixes. Dissertação (mestrado) - Universidade Estadual Paulista Júlio de Mesquita Filho, Centro de Aquicultura de Jaboticabal.

Verdouw, H., Vanechteld, C.J.A., Deckkers, E.M.J., 1978. Ammonia determinations based on indophenol formation with sodium salicylate. Water Research, 12, 399-402.

Ximenes R. M., *et al*. 2013. Antitumoral Activity of Leaves from *Hyptis mutabilis* (A.Rich.) Briq. (Lamiaceae) in Mice Bearing Tumor. Data set Papers in Pharmacology, Article ID 169357, 1-3.

Table 1. Biochemical serum parameters of healthy silver catfish after 1 h daily baths with OE of *Hyptis mutabilis* or (-)-globulol for four days, n=20.

Table 2. Hematological and immunological parameters of silver catfish after 1 h daily baths with EO of *Hyptis mutabilis* or (-)-globulol for four days, n=20.

Figure 1. Fish survival exposed to *Hyptis mutabilis* EO submitted to three different methodologies for four days: a single application at the beginning of the experiment (A), twice with an interval of 48 h (B) and 1 h daily baths (C). Fish survival after exposure to (-)-globulol through 1 h daily baths in four consecutive days (D). Comparison between EO 20 $mg.L^{-1}$ and (-)-globulol 2.5 $mg.L^{-1}$ (E). * Significant difference from control; #: Significant difference from the group treated with ethanol (Kaplan-Meier (Lag-Rank), $P \leq 0.01$), n=20.

Figure 2. Number of white spots in the dorsolateral region of silver catfish exposed to EO of *Hyptis mutabilis* submitted to three different methodologies for four days: a single application at the beginning of the experiment (A), twice with an interval of 48 h (B) and 1 h daily baths (C). Number of white spots in fish exposed to (-)-globulol through 1 h daily baths for four days (D). Lower case letters indicate significant differences between treatments evaluated in the same time. Capital letters indicate significant differences of the same treatment in different time periods ($P < 0.05$), n=20.

Table 1

Parameters	Treatment			
	Control	Ethanol	Globulol 2.5 $mg.L^{-1}$	EO 20 mgL^{-1}
Glucose ($mg.dL^{-1}$)	45.66 ± 3.48	48.33 ± 7.35	42.66 ± 0.88	48.33 ± 5.24
Albumin ($g.dL^{-1}$)	2.80 ± 0.35^{b}	4.17 ± 0.33^{a}	$2.96 \pm 0.18^{a,b}$	$3.30 \pm 0.20^{a,b}$
Total Bilirubin ($mg.dL^{-1}$)	0.16 ± 0.06	0.17 ± 0.02	0.23 ± 0.02	0.24 ± 0.05
Triglycerides ($mg\ dL^{-1}$)	192.66 ± 23.77	187.66 ± 11.89	185.66 ± 33.34	227.66 ±70.34
Total Proteins ($g.dL^{-1}$)	3.23 ± 0.18	3.00 ± 0.21	2.76 ± 0.26	4.33 ± 0.64
Total Cholesterol ($mg.dL^{-1}$)	193.66 ± 9.49^{b}	187.66 ± 5.36^{b}	183.00 ± 5.68^{b}	226.66 ± 5.48^{a}
HDL ($mg.dL^{-1}$)	148.00 ± 6.24^{a}	$119.66 \pm 19.17^{a,b}$	87.66 ± 4.81^{b}	$100.00 \pm 10.11^{a,b}$
LDL ($mg.dL^{-1}$)	98.00 ± 17.43^{b}	85.66 ± 10.98^{b}	$244.66 \pm 52.71^{a,b}$	463.00 ± 97.82^{a}
Creatinine ($mg.dL^{-1}$)	0.11 ± 0.006	0.09 ± 0.003	0.09 ± 0.04	0.09 ± 0.009
Urea ($mg.dL^{-1}$)	3.76 ± 0.93	2.96 ± 0.58	4.46 ± 0.48	4.76 ± 0.58

Table 2

Parameters	Treatment			
	Control	Ethanol	Globulol 2.5 $mg.L^{-1}$	OE 20 $mg.L^{-1}$
Hematocrit (%)	22.40 ± 0.73^{b}	$26.12 \pm 1.65^{a,b}$	$26.78 \pm 0.99^{a,b}$	28.42 ± 0.95^{a}
Hemoglobin (g%)	7.52 ± 0.48	7.07 ± 0.47	6.95 ± 0.29	7.34 ± 0.30
Erythrocytes (106 µL-1)	1.64 ± 0.07^{b}	2.01 ± 0.12^{a}	2.06 ± 0.08^{a}	2.11 ± 0.08^{a}
Leucocytes (10^3 μL^{-1})	12.01 ± 5.00^{c}	$57.61 \pm 10.00^{a,b}$	$70.87 \pm 15.00^{a,b}$	$48.82 \pm 7.00^{a,b,c}$
Thrombocytes (10^3 μL^{-1})	103.00 ± 12.00^{a}	95.80 ± 11.20^{a}	108.90 ± 12.30^{a}	20.50 ± 3.40^{b}
MCV (fL)	137.50 ± 4.45^{a}	$129.90 \pm 3.09^{a,b}$	129.87 ± 1.55^{b}	134.56 ± 1.29^{b}
MCHC ($g .L^{-1}$)	27.00 ± 0.11	33.95 ± 3.54	25.75 ± 0.33	25.95 ± 0.58
Monocytes (%)	10.66 ± 4.48	8.33 ± 3.52	9.33 ± 2.33	12.00 ± 0.57
Lymphocytes (%)	85.33 ± 4.91	86.33 ± 4.97	87.00 ± 2.51	85.33 ± 1.20
Heterophils (%)	3.33 ± 0.33	4.66 ± 1.45	3.33 ± 0.88	2.33 ± 0.88
Eosinophils (%)	0.66 ± 0.33	0.66 ± 0.33	0.33 ± 0.33	0.33 ± 0.33
Hemolysis of fish (%)	91.66 ± 3.08	88.69 ± 1.97	78.38 ± 3.88	83.44 ± 5.25

Figure 1

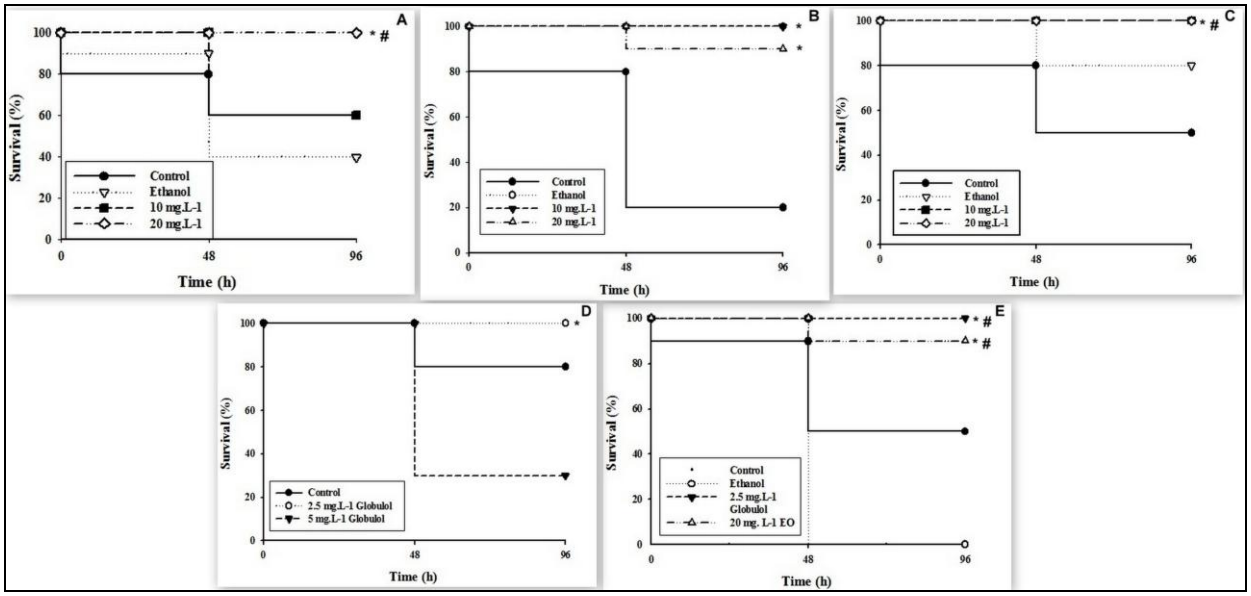
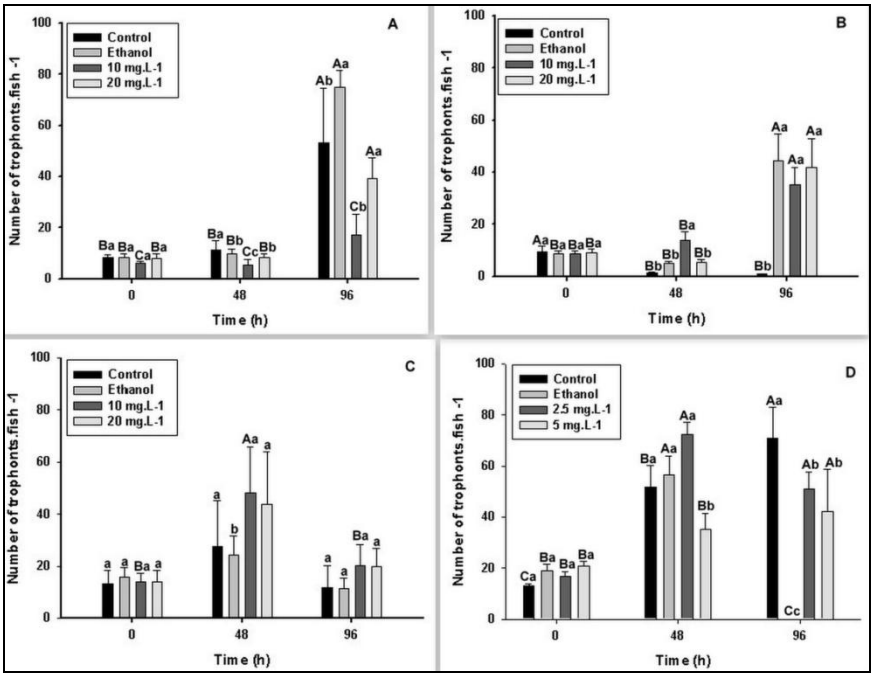

A
B
C
D
E
Survival (%)
Time (h)
Control
Ethanol
10 mg.L-1
20 mg.L-1
2.5 mg.L-1 Globulol
5 mg.L-1 Globulol
20 mg. L-1 EO

Figure 2


A
Control
Ethanol
10 mg.L-1
20 mg.L-1
Number of trophonts.fish -1
Time (h)
0
48
96
Ba Ba Ca Ba
Ba Bb Cc Bb
Ab Aa Cb Aa
B
Control
Ethanol
10 mg.L-1
20 mg.L-1
Number of trophonts.fish -1
Time (h)
Aa Ba Ba Ba
Bb Bb Ba Bb
Bb Aa Aa Aa
C
Control
Ethanol
10 mg.L-1
20 mg.L-1
Number of trophonts.fish -1
Time (h)
a a Ba a
a b Aa a
a a Ba a
D
Control
Ethanol
2.5 mg.L-1
5 mg.L-1
Number of trophonts.fish -1
Time (h)
Ca Ba Ba Ba
Ba Aa Aa Bb
Aa Cc Ab Ab

4 CONCLUSÃO

- O óleo essencial (OE) de folhas de *Hyptis mutabilis* e constituinte (-)-globulol não foram eficazes na diminuição do número de trofontes decorrentes da parasitose por *Ichthyophthirius multifiliis* em jundiás;

- Dentre as três metodologias avaliadas, a metodologia que apresentou melhor desempenho considerando-se a sobrevivência dos animais parasitados foi banhos diários de 1 hora por 4 dias consecutivos;

- A sobrevivência dos animais parasitados com *Ichthyophthirius multifiliis* foi aumentada quando os mesmos foram expostos ao OE (20 $mg.L^{-1}$) e ao seu constituinte majoritário, (-)-globulol (2,5 $mg.L^{-1}$) através de banhos diários de 1h por 4 dias consecutivos;

- O OE de *Hyptis mutabilis* e o (-)-globulol não inibiram a atividade hemolítica de *A. hydrophila* em eritrócitos de peixes sadios;

- A adição do OE de *Hyptis mutabilis* e do (-)-globulol à água não apresentou correlação clara entre a alteração dos parâmetros hematológicos e bioquímicos em jundiás sadios e o aumento da sobrevivência de parasitados.

REFERÊNCIAS BIBLIOGRÁFICAS

AOKI, T. *et al*. Molecular innate immunity in teleost fish? Review and future perspectives. **Fish Glob Welf Environ,** p. 263-276, 2008. Available on: http://www.terrapub.co.jp/onlineproceedings/fs/wfc2008/pdf/wfcbk_263.pdf. Accessed: September 19, 2014.

BALDISSEROTTO, B. **Fisiologia de peixes aplicada à piscicultura.** 2.ed. Santa Maria: Universidade Federal de Santa Maria, p. 350, 2009.

BARCELLOS, L. J. G. *et al*. Hematological changes in jundiá (*Rhamdia quelen* Quoy and Gaimard Pimelodidae) after acute and chronic stress caused by usual aquacultural management, with emphasis on immunosuppressive effects. **Aquaculture,** v. 237, p. 229–236, 2014.

BRASIL. Ministério da Pesca e Aquicultura. Aquicultura, Espécies Cultivadas. Setembro de 2014. Disponível em: <http://www.mpa.gov.br/index.php/aquicultura/especies-cultivadas>. Acesso em: 10 nov. 2014.

BRASIL. Ministério da Pesca e Aquicultura. Aquicultura, Produção. Julho de 2014. Disponível em: <http://www.mpa.gov.br/index.php/aquicultura/producao>. Acesso em: 10 nov. 2014.

BORDIGNON, S. A. L. **O Gênero *Hyptis* Jacq. (Labiatae) no Rio Grande do Sul.** Tese de Mestrado, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, 1990.

BUCHMANN, K.; LINDENSTROM, T.; SIGH, J. Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjav* in iimmunized rainbow trout. **J. Helminthol**, v. 73, p. 189–195, 1999.

BUCHMANN, K. *et al*. Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*. **Vet. Parasitol**, v. 100, p. 105–116, 2001.

CARNEIRO, P. C. F.; SCHORER, M.; MIKOS. J. D. Tratamentos terapêuticos convencionais no controle do ectoparasito *Ichthyophtirius multifiliis* em jundiá (*Rhamdia quelen*). **Pesq. Agropec. Bras**.v. 40, p. 99–102, 2005.

CIGANA, C. **Piscicultura é alternativa para aumentar a rentabilidade das propriedades.** Zero Hora, 2014. Disponível em: <file:///C:/Documents%20and%20Settings/pccli/Meus%20documentos/Downloads/O%20Rio%20Grande%20est%C3%83%C2%A1%20para%20peixe%20-%20Campo%20e%20Lavoura%20-%20Not%C3%83-cias%20-%20Zero%20Hora.html>. Acesso em: 29 out. 2014.

CEPAL/ FAO/ IICA. **Perpectivas de la agricultura y del desarollo rural em lãs Americas: uma mirada hacia América Latina y el Caribe, 2011-2012**. San José, Costa Rica, p. 182, 2011.

CONROY, D. A.; VASQUEZ, D. C. Las principales enfermidades infectocontagiosas de los salmonidos. Bogotá: Projecto Desarollo de la Pesca Continental INDERENA/FAO, p. 252, 1975.

CURTIS, C. *et al*. Natural and synthetic repellents. In: CURTIS, C.F. Editor. **Appropriate Technology in Vector Control**. CRC Press, Florida. p. 76–89, 1989.

DICKERSON, H. W. *Ichthyophthirius multifiliis* and *Cryptocary onirritans* (phylum Ciliophora). In: Woo, P.T.K. (Ed.), **Fish Diseases and Disorders**, v. 1. Protozoanand metazoan infections, 2nd ed. CAB International, Wallingford, UK, p. 116–153, 2006.

DICKERSON, H. W. *Ichthyophthirius multifiliis*. In: PATRICK, K. B., WOO, T. K. (Eds.), **Fish Parasites Pathobiology and Protection**. CABI, Wallingford, p. 55–72, 2012.

FACHINETTO, J. M.; TEDESCO, S. B. Número cromossômico, análise meiótica e estimativa da viabilidade polínica em populações de *Hyptis mutabilis* (Rich.) Briq. **Rev. Bras. Pl. Med**., Botucatu, v. 11, n. 1, p. 110-116, 2009.

FAIRFIELD, T. A Commonsense guide to fish health. **Barrons**, New York, NY; p.134, 2000.

FAO. Fishery and Aquaculture Statistics. FAO Yearbook, 2009. FAO Fisheries Department. **Food and Agriculture Organization**, Rome, p. 80, 2011.

FAO. The State of World Fisheries and Aquaculture 2010. FAO Fisheries Department. **Food and Agriculture Organization**, p. 197, Rome, 2010.

FERREIRA, V. F. ; PINTO, A. C. A fitoterapia no mundo atual. **Quím. Nova,** v.33, n.9, São Paulo, 2010**.**

FRACALOSSI, D. M.; ZANIBONI, F. E.; MEURER, S. No rastro das espécies nativas. **Panorama Aquicultura**, São Paulo, v. 12, n. 74, p. 43-49, 2002.

GARCIA, L. D. *et al*. Salt in the food and water as a supportive therapy for *Ichthyophthirius multifiliis* infestation on silver catfish, *Rhamdia quelen*, fingerlings. **Journal of the World Aquaculture Society,** v. 38, n. 1, 2007.

GARCIA, L. D. *et al*. Effects of water pH and hardness on infection of silver catfish, *Rhamdia quelen*, fingerlings by *Ichthyophthirius multifiliis*. **Journal of the World Aquaculture Society,** v. 42, n. 3, p. 399-405, 2011.

GHIRALDELL, L. *et al*. Infeccção com trofozoítos de *Ichthyophthirius multifiliis* (CILIOPHORA) em *Poecilia vivipara* (POECILIIDAE) como hospedeiro experimental. **Ciência Animal Brasileira**, v. 8, n. 1, p. 105-110, 2007.

GILLIJ, G. Y.; GLEISER R. M.; ZYGADLO, J. A. Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. **Bioresource Technology,** v. 99, p. 2507–2515, 2008.

GOMES, L. C. *et al*. Biologia do jundiá Rhamdia quelen (TELEOSTEI, PIMELODIDAE). **Ciênc. Rural**, 30, 179-185, 2000.

GUEDES, D. S. **Contribuição ao estudo da sistemática e alimentação de jundiás (*Rhamdia* spp.) na região central do Rio Grande do Sul (Pisces, Pimelodidae)**. Santa Maria – RS, 1980, p. 99. Dissertação (Mestrado em Zootecnia) - Curso de Pós-graduação em Zootecnia, Universidade Federal de Santa Maria, 1980.

INFOPESCA. 32ª Conferência Regional da FAO Buenos Aires, Argentina, 26 a 30 de março de 2012. **Comunicação oral por Roland Wiefels** (Diretor INFOPESCA).

KREUTZ, L. C. *et al*. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. **Fish and Shellfish Immunology**, v. 29, n. 4, p. 694-697, 2010.

LAZZARI, R. *et al*. Hematologia de juñidas em resposta ao nível de proteína. **Ci. Aim. Bras**., Goiania. v. 12, n. 2, p. 192-197, 2011.

LYNN, D. H. **The ciliated protozoa characterization, classification, and guide to the literature**. 3 ed. Springer, Heidelberg, London, New York, p. 605, 2008.

MANSKE, O. **Viabilidade econômica da produção do jundiá cinza (*Rhamdia quelen*) em tanques-rede na região de Vacaria-RS.** Portal dos Profissionais da Biologia, 2009. Disponível em: <file:///C:/Documents%20and%20Settings/pccli/Meus%20documentos/Downloads/Artigo%203577313433%20de%20CRBio%20075636%2003%20-%20Cat%C3%83%C2%A1logo%20dos%20Profissionais%20do%20CRBIO%20-.html>. Acesso em: 29 out. 2014.

MARTINS, M. L. *et al*. Recent studies on parasitic infections of freshwater cultivated fish in the State of São Paulo, Brazil. **Acta Scientiarum**, Maringá, v. 24, n. 4, p. 981-985, 2002.

MATTHEWS, R. A. *Ichthyophthirius multifiliis* Fouquet and Ichthyophthiriosis in freshwater teleosts. **Adv. Parasitol.,** v. 59, p. 159-241, 2005.

NERIO, L. S.; JESUS, O. V.; STASHENKO, E. Repellent activity of essential oils: Areview. **Bioresour.Technol,** v. 101, p. 372-378, 2010.

Ostrensky, **A. Estudos para viabilização tecnológica dos cultivos de camarões marinhos no litoral do Estado do Paraná, Brasil.** 1997. 126 p. Tese de doutorado. Universidade Federal do Paraná, Curitiba, PR. Brasil. 1997.

ORGANIZAÇÃO Mundial da Saúde (OMS). **Alma-Ata 1978 - Cuidados primários de saúde**. Relatório da conferência internacional sobre cuidados primários de saúde. Brasília: Organização Mundial da Saúde/Fundo das Nações Unidas para a Infância; 1979.

PERGENTINO DE SOUZA, D.; FARIAS NÓBREGA, F. F.; ALMEIDA, R. N. Influence of the chirality of (*R*)-(-)- and (*S*)-(+)-carvone in the central nervous system: a comparative tudy. **Chirality,** v. 19, p. 264-268, 2007.

PICÓN-CAMACHO, S. M. P. M. *et al*. An assessment of the use of drug and non-drug interventions in the treatment of *Ichthyophthirius multifiliis* Fouquet, 1876, a protozoan parasite of fresh water fish. **Parasitology**, v. 139, p. 149-190, 2012.

POST, G. **Textbook of fish health.** TFH Publications. Neptune City, p. 288, 1987.

REZENDE, H. A. ; COCCO, M. I. M. A utilização de fitoterapia no cotidiano de uma população rural. **Rev. Esc. Enferm.** v. 36, n. 3, São Paulo, 2002.

SEO, K. A. *et al.* The monoterpenoids citral and geraniol are moderate inhibitors of CYP2B6 hydroxylase activity. **Chemico-Biological Interactions,** v. 1744, p. 141-146, 2008.

SILFVERGRIP, A. M. C. A sistematic revision of the neotropical catfish genus *Rhamdia* (Teleostei, Pimelodidae). Stockholm, Sweden. PhD Thesis - Department of Zoology, Stockholm University and Department of Vertebrate Zoology, Swedish Museum of Natural History, 1996.

SILVA, L. L. *et al*. Sedative and anesthetic activities of the essential oils of *Hyptis mutabilis* (Rich.)Briq. and their isolated components in silver catfish (*Rhamdia quelen*). **Brazilian Journal of Medical and Biological Research**, v. 46, p. 771-779, 2013.

SWARÇA, A. C.; GIULIANO-CAETANO, L.; DIAS, A. L. Cytogeneticsof species of the families Pimelodidae and Rhamdiidae (Siluriformes). **Genet. Mol. Biol.** v. 23, p. 589-593, 2000.

STOSKOPF, M. K. **Fish Medicine**. W. B Saunders Co. Philadelphia, PA; 1993.

SUTILI, F. J. *et al*. The use of nitazoxanide against the pathogens *Ichthyophthirius multiliis* and *Aeromonas hydrophila* in silver catfish (Rhamdia quelen). **Vet. Parasitol.** (Print), p. 522-526, 2013.

SUTILLI, F. J., *et al*. The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*). **Veterinary Immunology and Immunopathology,** v. 157, p. 142-148, 2014.

TAVARES-DIAS, M.; SCHALCH, S. H. C.; MARTINS, M. L. Características hematológicas de *Oreochromisn iloticus* (Osteichthyes: Cichlidae) cultivadas intensivamente em "Pesque-Pague" do Município de Franca, São Paulo, Brasil. **Ars Veterinaria**, Jaboticabal, v. 16, n. 2, p. 76-82, 2000.

VALLADÃO, G. M. R. **Potencial de óleos essenciais de plantas para o tratamento de enfermidade em peixes.** (Dissertação) Universidade Federal Paulista "Júlio de Mesquita Filho". Jabotical – São Paulo, 2014.

VARGAS, R. J. Resposta fisiológica à infestação experimental com *Ichthyophthirius multifiliis* (Fouquet, 1876) em alevinos de jundiá (*Rhamdia quelen* Guoy e Gaimard, 1824) previamente alimentados com diferentes fontes lipídicas. **Rev. Bras. Parasitol. Vet.,** v. 17, n. 2, p. 81-86, 2008.

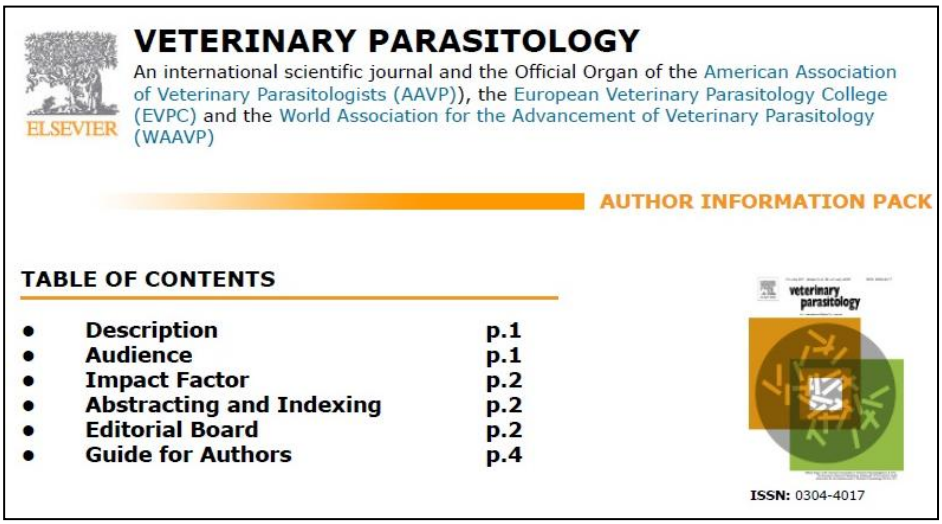
WHYTE, S. The innate immune response of finfish - a review of current knowledge. **Fish and Shellfish Immunology**, v. 23, n. 6, p. 1127-1151, 2007.

XIMENES R. M., *et al*. Antitumoral activity of leaves from *Hyptis mutabilis* (A.Rich.) Briq. (Lamiaceae) in mice bearing tumor. **Data set Papers in Pharmacology**, v.2013, p. 1-3, 2013.

ANEXO

Anexo A - Normas para publicação no periódico Veterinary Parasitology

VETERINARY PARASITOLOGY



VETERINARY PARASITOLOGY

An international scientific journal and the Official Organ of the American Association of Veterinary Parasitologists (AAVP)), the European Veterinary Parasitology College (EVPC) and the World Association for the Advancement of Veterinary Parasitology (WAAVP)

ELSEVIER

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0304-4017

DESCRIPTION

This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology. Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology, and control of parasites in all domesticated animals, fall within the scope of the journal. Papers of geographically limited (local) interest which are not of interest to an international audience will not be accepted. Authors who submit papers based on local data will need to indicate why their paper is relevant to a broader readership.

Parasitological studies on laboratory animals fall within the scope of the journal only if they provide a reasonably close model of a disease of domestic animals. Additionally the journal will consider papers relating to wildlife species where they may act as disease reservoirs to domestic animals, or as a zoonotic reservoir. Case studies considered to be unique or of specific interest to the journal, will also be considered on occasions at the Editors' discretion. Papers dealing exclusively with the taxonomy of parasites do not fall within the scope of the journal.

Studies on rickettsial disease organisms (Ehrlichia, Anaplasma, Eperythrozoon) will be considered for publication in Veterinary Parasitology, but only if the paper deals with vector

transmission of these organisms to domesticated animals, or if zoonotic. Studies on Rickettsia per se will not be accepted.

Studies dealing with parasite control by means of natural products, both in vivo and in vitro, fall within the scope of the journal, but only if well documented and with therapeutically relevant minimum inhibitory concentrations of the active compound(s) being clearly demonstrated.

Circumstances relating to animal experimentation must meet the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for International Organizations of Medical Sciences. (Obtainable from: Executive Secretary C.I.O.M.S., c/o W.H.O., Via Appia, CH-1211
Geneva 27, Switzerland.)

AUDIENCE

Research Workers and Practitioners in veterinary medicine, Animal Health Investigators and others with a special interest in parasitology, veterinary pharmaceutical industry.

IMPACT FACTOR

ABSTRACTING AND INDEXING

BIOSIS
Elsevier BIOBASE
Current Contents/Agriculture, Biology & Environmental Sciences
Index Catalog of Medical and Veterinary Zoology
MEDLINE®
Index Veterinarius Helminthological Abstracts Protozoological Abstracts
Referativnyi Zhurnal VINTI-RAN (Russian Academy of Sciences) Veterinary Bulletin
Scopus

EDITORIAL BOARD

Editors-in-Chief

T. de Waal, Veterinary Science Centre, University College Dublin, Ireland A.A. Gajadhar, Canadian Food Inspection Agency, Saskatoon, SK, Canada C. Genchi, DIPAV, Faculty of Veterinary Medicine, Milan, Italy

D.K. Howe, Dept of Veterinary Science, M.H. Gluck Equine Research Center, University of Kentucky, Lexington, KY USA

M.P. Reichel, University of Adelaide, Adelaide, SA, Australia

Book Review Editor

W.E. Pomroy, Palmerston North, New Zealand

Editorial Advisory Board

L. R. Ballweber, Fort Collins, CO, USA

G. Baneth, Rehovot, Israel

D. Berkvens, Antwerp, Belgium R.B. Besier, Albany, WA, Australia J. Boes, Skejby, Denmark

D.D. Bowman, Ithaca, NY, USA

E. Claerebout, Merelbeke, Belgium

P. Deplazes, Zürich, Switzerland

P. Dorchies, Toulouse, France

P. Dorny, Antwerp, Belgium

H.M. Elsheikha, Leicestershire, UK

S. Geerts, Antwerp, Belgium

J. Gilleard, Calgary, AB, Canada

P.J. Holman, College Station, TX, USA

I. Igarashi, Hokkaido, Japan D.P. Jasmer, Pullman, WA, USA R. Kaplan, Athens, GA, USA

T.J. Kennedy, Phoenix, AZ, USA A.C. Kotze, St. Lucia, QLD, Australia L.H. Kramer, Parma, Italy

R.C. Krecek, College Station, TX, USA D.S. Lindsay, Blacksburg, VA, USA L.S. Mansfield, East Lansing, MI, USA

A.A. Marchiondo, Kalamazoo, MI, USA

M.B. Molento, Curitiba, Brazil

V.P.M.G. Naidoo, Pretoria, South Africa

M.K. Nielsen, Lexington, KY, USA T.J. Nolan, Philadelphia, PA, USA P.U. Olafson, Kerrville, TX, USA D. Otranto, Bari, Italy
E. Papadopoulos, Thessaloniki, Greece
K. Pfister, München, Germany
L. Polley, Saskatoon, SK, Canada
W.E. Pomroy, Palmerston North, New Zealand
D.E. Rebeski, Cuxhaven, Germany
M.G. Rossano, Lexington, KY, USA
M. Santin-Duran, Beltsville, MD, USA
T. Schetters, Boxmeer, Netherlands
J. Schröder, North Sydney, NSW, Australia
V. Shkap, Bet Dagan, Israel
J. Šlapeta, New South Wales, Australia
D.E. Snyder, Greenfield, IN, USA R.W. Stich, Columbia, MO, USA B.E. Stromberg, St Paul, MN, USA M.A. Taylor, Market Weighton, UK
R.C.A. Thompson, Murdoch, WA, Australia
J. Vercruysse, Ghent, Belgium
G. Von Samson-Himmelstjerna, Berlin, Germany
D. Zarlenga, Beltsville, MD, USA
A. Zintl, Dublin, Ireland

WAAVP Society Members
R.C. Krecek, Basseterre, Saint Kitts and Nevis
J. Vercruysse, Ghent, Belgium

GUIDE FOR AUTHORS

INTRODUCTION

Types of contribution

1. Original research papers (Regular Papers)
2. Review articles
3. Rapid Communications
4. Short Communications
5. Letters to the Editor

6. Book Reviews

Original research papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review articles should cover subjects falling within the scope of the journal which are of active current interest. They may be submitted or invited.

Rapid Communications should contain information of high 'news'/scientific value worthy of very rapid publication. Rapid Communications should be submitted to the journal as such (i.e. clearly labelled as a RC) and should, in general, not exceed 2000 words in length. Upon receipt, they will be subject to rapid assessment and if accepted, published with priority.

Short Communications should consist of original observations or new methods within the scope of the journal. Reports of observations previously published from different geographical areas may be accepted only if considered sufficiently unusual or noteworthy. The Communications should be concise with the minimum of references, and cover no more than four pages of the journal; they need not be formally structured as are full papers, but should give sufficient methods and data necessary for their comprehension.

Letters to the Editor offering comment or useful critique on material published in the journal are welcomed. The decision to publish submitted letters rests purely with the Editors-in-Chief. It is hoped that the publication of such letters will permit an exchange of views which will be of benefit to both the journal and its readers.

Book Reviews will be included in the journal on a range of relevant books which are not more than 2 years old and were written in English.

Book reviews will be solicited by the Book Review Editor. Unsolicited reviews will not usually be accepted, but suggestions for appropriate books for review may be sent to the Book Review Editor:

Dr W. Pomroy
Institute of Veterinary, Animal and Biomedical Sciences
Massey University
Private Bag 11 222
Palmerston North 4442
New Zealand w.pomroy@massey.ac.nz

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Ethics

Circumstances relating to animal experimentation must meet the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences. They are obtainable from: Executive Secretary C.I.O.M.S., c/o WHO, Via Appia, CH-1211 Geneva 27, Switzerland, or at the following URL: http://www.cioms.ch/publications/ guidelines/1985_texts_of_guidelines.htm. Unnecessary cruelty in animal experimentation is not acceptable to the Editors of Veterinary Parasitology.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Please include this under a heading "Conflict of interest statement" at the end of the text.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other

language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

This journal offers authors a choice in publishing their research: Open access and Subscription.

For subscription articles

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

For open access articles

Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Retained author rights

As an author you (or your employer or institution) retain certain rights. For more information on author rights for:

Subscription articles please see http://www.elsevier.com/journal-authors/author-rights-and-responsibilities.

Open access articles please see http://www.elsevier.com/OAauthoragreement.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal offers authors a choice in publishing their research:

Open access

• Articles are freely available to both subscribers and the wider public with permitted reuse

• An open access publication fee is payable by authors or their research funder

Subscription

• Articles are made available to subscribers as well as developing countries and patient groups through our access programs (http://www.elsevier.com/access)

• No open access publication fee

All articles published open access will be immediately and permanently free for everyone to read and download. Permitted reuse is defined by your choice of one of the following Creative Commons user licenses:

Creative Commons Attribution-NonCommercial-ShareAlike (CC BY-NC-SA): for non-commercial purposes, lets others distribute and copy the article, to create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, do not modify the article in such a way as to damage the author's honor or reputation, and license their new adaptations or creations under identical terms (CC BY-NC-SA).

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND): for non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

Elsevier has established agreements with funding bodies, http://www.elsevier.com/fundingbodies. This ensures authors can comply with funding body open access requirements, including specific user licenses, such as CC BY. Some authors may also be reimbursed for associated publication fees. If you need to comply with your funding body policy, you can apply for the CC BY license after your manuscript is accepted for publication.

To provide open access, this journal has a publication fee which needs to be met by the authors or their research funders for each article published open access.
Your publication choice will have no effect on the peer review process or acceptance of submitted articles.
The open access publication fee for this journal is US$ 2500, excluding taxes. Learn more about
Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Authors should also be aware that The Lucidus Consultancy edit@luciduscconsultancy.com offers a bespoke service to putative contributors to Veterinary Parasitology who need to arrange language improvement for their manuscripts.

Please note that Elsevier neither endorses nor takes responsibility for any products, goods or services offered by outside vendors through our services or in any advertising. For more information please refer to our terms & conditions http://www.elsevier.com/termsandconditions.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

A cover letter is required for each new submission. It should address the novelty and significance of the work and how it fits within the defined scope of Veterinary Parasitology. Essential information, issues of concern or potential problems, (such as other publications or submissions containing similar information) should be identified in the cover letter. Authors who submit papers based on local data/ surveys will need to indicate why their paper is relevant to a broader readership.

Authors are invited to suggest the names of up to 5 referees (with email addresses) whom they feel are qualified to evaluate their submission. Submission of such names does not, however, imply that they will definitely be used as referees.

Authors should send queries concerning the submission process or journal procedures to AuthorSupport@elsevier.com. Authors can check the status of their manuscript within the review procedure using Elsevier Editorial System.
Authors submitting hard copy papers will be asked to resubmit using Elsevier Editorial System. Submission of an article is understood to imply that the article is original and is not being considered for publication elsewhere. Submission also implies that all authors have approved the paper for release and are in agreement with its content. Upon acceptance of the article by the journal, the author(s) will be asked to transfer the copyright of the article to the Publisher. This transfer will ensure the widest possible dissemination of information.

Article Transfer Service
This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. More information about this can be found here: http://www.elsevier.com/authors/article-transfer-service.

Submit your article

Please submit your article via http://ees.elsevier.com/vetpar.

PREPARATION

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word

processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Manuscripts should have numbered lines with wide margins and double spacing throughout, i.e. also for abstracts, footnotes and references. Every page of the manuscript should be numbered. However, in the text no reference should made to page numbers; if necessary, one may refer to sections. Avoid excessive usage of italics to emphasize part of the text.

Manuscripts in general should be organized in the following order: Title (should be clear, descriptive and not too long)

Name(s) of author(s)

Complete postal address(es) of affiliations

Full telephone, Fax No. and e-mail address of the corresponding author

Present address(es) of author(s) if applicable

Complete correspondence address including e-mail address to which the proofs should be sent

Abstract

Keywords (indexing terms), normally 3-6 items. Please refer to last index (Vol. 100/3-4).

Introduction

Material studied, area descriptions, methods, techniques

Results Discussion Conclusion

Acknowledgments and any additional information concerning research grants, etc.

References

Tables

Figure captions

Tables (separate file(s)) Figures (separate file(s)).

Titles and subtitles should not be run within the text. They should be typed on a separate line, without indentation. Use lower-case letter type.

SI units should be used.

Elsevier reserves the privilege of returning to the author for revision accepted manuscripts and illustrations which are not in the proper form given in this guide.

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered

1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full

postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.

• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract should be clear, descriptive and not more than 400 words.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 ×

13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Formulae

1. Give the meaning of all symbols immediately after the equation in which they are first used.

2. For simple fractions use the solidus (/) instead of a horizontal line.

3. Equations should be numbered serially at the right-hand side in parentheses. In general only equations explicitly referred to in the text need be numbered.

4. The use of fractional powers instead of root signs is recommended. Powers of e are often more conveniently denoted by exp.

5. In chemical formulae, valence of ions should be given as, e.g. Ca2+, not as Ca++.

6. Isotope numbers should precede the symbols e.g. 18O.

7. The repeated use of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g. phosphate as P2O5).

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature

1. Authors and editors are, by general agreement, obliged to accept the rules governing biological nomenclature, as laid down in the International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria, and the International Code of Zoological Nomenclature.

2. All biotica (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals.

3. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.

4. For chemical nomenclature, the conventions of the International Union of Pure and Applied Chemistry and the official recommendations of the IUPAC-IUB Combined Commission on Biochemical Nomenclature should be followed.

5. For the denomination of parasitic diseases or infections, authors are advised to consult the Standardized Nomenclature of Animal Parasitic Diseases (SNOAPAD) published in 1988 in Veterinary Parasitology (Kassai, T. et al., 1988. Vet. Parasitol. 29, 299-326).

Submission of sequence data to databases

New nucleotide or amino acid sequence data must be deposited in publicly accessible databases, such as GenBank™, EMBL or DDJB, and an accession number obtained and submitted to the Publisher (at the latest) together with the final, revised manuscript. The accession number should appear in a separate paragraph in the Materials and Methods section (example: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers: XXXX, XXXX). In order for automatic links to be made between papers and databases, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalised. When published they will appear in normal type.

Elsevier encourages authors to connect articles with external databases, giving their readers one- click access to relevant databases that help to build a better understanding of the described research. Please refer to http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork Electronic artwork General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Size the illustrations close to the desired dimensions of the printed version.

• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications that can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Illustration services

Elsevier's WebShop (http://webshop.elsevier.com/illustrationservices) offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical- style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Tables

1. Authors should take notice of the limitations set by the size and lay-out of the journal. Large tables should be avoided. Reversing columns and rows will often reduce the dimensions of a table.

2. If many data are to be presented, an attempt should be made to divide them over two or more tables.

3. Tables should be numbered according to their sequence in the text. The text should include references to all tables.

4. Each table should occupy a separate page of the manuscript. Tables should never be included in the text.

5. Each table should have a brief and self-explanatory title.

6. Column headings should be brief, but sufficiently explanatory. Standard abbreviations of units of measurement should be added between parentheses.

7. Vertical lines should not be used to separate columns. Leave some extra space between the columns instead.
8. Any explanation essential to the understanding of the table should be given as a footnote at the bottom of the table.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or
'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference Style

1. All publications cited in the text should be presented in a list of references following the text of the manuscript. The manuscript should be carefully checked to ensure that the spelling of author's names and dates are exactly the same in the text as in the reference list.

2. In the text refer to the author's name (without initial) and year of publication, followed – if necessary
– by a short reference to appropriate pages. Examples: "Since Peterson (1988) has shown that..." "This is in agreement with results obtained later (Kramer, 1989, pp. 12–16)".

3. If reference is made in the text to a publication written by more than two authors the name of the first author should be used followed by "et al.". This indication, however, should never be used in the list of references. In this list names of first author and co-authors should be mentioned.

4. References cited together in the text should be arranged chronologically. The list of references should be arranged alphabetically on author's names, and chronologically per author. If an author's name in the list is also mentioned with co-authors the following order should be used: publications of the single author, arranged according to publication dates – publications of the same author with one co-author – publications of the author with more than one co-author. Publications by the same author(s) in the same year should be listed as 1974a, 1974b, etc.

5. Use the following system for arranging your references:

a. For periodicals

Lanusse, C.E., Prichard, R.K., 1993. Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics. Vet. Parasitol. 49, 123–158.

b. For edited symposia, special issues, etc., published in a periodical

Weatherley, A.J., Hong, C., Harris, T.J., Smith, D.G., Hammet, N.C., 1993. Persistent efficacy of doramectin against experimental nematode infections in calves. In: Vercruysse, J. (Ed.), Doramectin– a novel avermectin. Vet. Parasitol. 49, 45–50. c. For books Blaha, T. (Ed.), 1989. Applied Veterinary Epidemiology. Elsevier, Amsterdam, 344 pp. d. For multi-author books

Wilson, M.B., Nakane, P.K., 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp, W., Holubar, K., Wick, G. (Eds.), Immunofluorescence and Related Staining Techniques. North Holland, Amsterdam, pp. 215–224.

6. Abbreviate the titles of periodicals mentioned in the list of references in accordance with BIOSIS Serial Sources, published annually by BIOSIS. The correct abbreviation for this journal is Vet. Parasitol.

7. In the case of publications in any language other than English, the original title is to be retained. However, the titles of publications in non-Latin alphabets should be transliterated, and a notation such as "(in Russian)" or "(in Greek, with English abstract)" should be added.

8. Work accepted for publication but not yet published should be referred to as "in press".

9. References concerning unpublished data and "personal communications" should not be cited in the reference list but may be mentioned in the text.
10. Web references may be given. As a minimum, the full URL is necessary. Any further information, such as Author names, dates, reference to a source publication and so on, should also be given.

11. Articles available online but without volume and page numbers may be referred to by means of their Digital Object identifier (DOI) code.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations:
http://www.issn.org/services/online-services/access-to-the-ltwa/.

AudioSlides
The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data
Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high- resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more

detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes) Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the

Internet)

Printed version of figures (if applicable) in color or black-and-white

• Indicate clearly whether or not color or black-and-white in print is required.

• For reproduction in black-and-white, please supply black-and-white versions of the figures for printing purposes.

For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes.

Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal Physics Letters B):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor.It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Authors can also keep track of the progress of their accepted article, and set up e-mail alerts informing them of changes to their manuscript's status, by using the "Track your accepted article" option on the journal's homepage http://www.elsevier.com/locate/vetpar For privacy, information on each article is password-protected. The author should key in the "Our Reference" code (which is in the letter of acknowledgement sent by the Publisher on receipt of the accepted article) and the name of the corresponding author.

Offprints

The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article on ScienceDirect. This link can

also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

AUTHOR INQUIRIES

You can track your submitted article at http://help.elsevier.com/app/answers/detail/a_id/89/p/8045/. You can track your accepted article at http://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.

© Copyright 2014 Elsevier | http://www.elsevier.com