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ÓLEOS ESSENCIAIS COMO ANESTÉSICOS EM SERRALMÍDEOS E EXPRESSÃO GÊNICA DE GENES RELÓGIO E DAS ENZIMAS DIGESTIVAS EM JUNDIÁ

TESE DE DOUTORADO

Ana Paula Gottlieb Almeida

Santa Maria, RS, Brasil
2018

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RESUMO

ÓLEOS ESSENCIAIS COMO ANESTÉSICOS EM SERRASALMÍDEOS E EXPRESSÃO GÊNICA DE GENES RELÓGIO E DAS ENZIMAS DIGESTIVAS EM JUNDIÁ

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Data e Local da Defesa: Santa Maria, 31 de agosto de 2018.

Anestésicos de origem sintética são amplamente empregados na aquicultura para minimizar o estresse decorrente das práticas envolvidas nessa atividade. No entanto, os efeitos adversos causados por essas substâncias sintéticas têm incentivado a pesquisa por alternativas naturais com propriedades sedativas e anestésicas que apresentem maior eficácia, segurança e menor risco de efeitos adversos. Estudos comprovaram que os óleos essenciais (OEs) extraídos das espécies vegetais *Aloysia triphylla* e *Lippia alba*, bem como o eugenol, um composto isolado do OE de cravo, apresentam propriedades sedativas e anestésicas em peixes, sendo considerados, portanto, alternativas promissoras aos anestésicos tradicionais. Nesse contexto, a primeira parte deste estudo objetivou investigar os efeitos desses OEs em duas espécies amazônicas, *Serrasalmus rhombeus* e *S. eigenmanni*, habitantes do Rio Negro. Já na segunda parte, avaliou-se a expressão gênica de genes relógio e de enzimas digestivas em pós-larvas e juvenis de *Rhamdia quelen*. O artigo 1 determinou a eficácia sedativa e anestésica dos OEs de *A. triphylla* e de *L. alba* e do eugenol em *S. rhombeus* e o tempo de recuperação dos animais, bem como comportamento natatório em uma exposição curta de 15 min em concentrações baixas para avaliar o possível uso dessas substâncias no transporte dessa espécie. Ambos OEs e o eugenol são eficazes, sendo recomendadas as concentrações de 150, 200 e 50 µL L^{-1}, respectivamente, para anestesia. Para a sedação dessa espécie é recomendada a utilização de 50 µL L^{-1} para ambos OEs e 25 µL L^{-1} para o eugenol. As concentrações de 5 e 10 µL L^{-1} são indicadas para estudos de transporte com esses anestésicos. No manuscrito 2 foi determinada a eficácia sedativa e anestésica dos OEs de *A. triphylla* e de *L. alba* em *S. eigenmanni*, bem como o tempo de recuperação dos animais. Avaliou-se também o fluxo iônico líquido, parâmetros sanguíneos e o comportamento natatório em uma exposição prolongada de 4 h. Ambos OEs foram eficazes para anestesia de *S. eigenmanni*, sendo recomendada a concentração de 100 µL L^{-1} para ambos os OEs e para a sedação as concentrações indicadas são 25 e 50 µL L^{-1} de OEs de *A. triphylla* e de *L. alba*, respectivamente. Para o transporte sugere-se a concentração de 5 µL L^{-1} do OE de *A. triphylla* devido à diminuição da excreção de amônia e à manutenção do equilíbrio. No manuscrito 3, foi avaliado a expressão gênica de genes relógio e de enzimas digestivas em pós-larvas e juvenis de *R. quelen*. Em pós-larvas, alguns genes da alça positiva (*artnl1a* e *clock*) e da alça negativa (*per1,2,3* e *cry1,2*) apresentaram diferenças nos níveis de expressão ao longo do período avaliado, sendo que a acrofase ocorreu no mesmo horário. Em juvenis, um gene da alça positiva (*arntl1l*) e um gene da alça negativa (*cry2*) apresentaram diferenças nos níveis de expressão, com diferença de 1 h entre as acrófases. Os níveis de expressão gênica das enzimas digestivas em pós-larvas foram máximos 4 h antes da alimentação e mínimos 1 h após a alimentação. No trato gastrintestinal de juvenis os níveis de expressão gênica das enzimas digestivas não diferiu no período avaliado. Em conclusão, ambos os OEs são eficazes para sedação e anestesia de *S. rhombeus* e de *S. eigenmanni*, podendo também serem utilizados em estudos de transportes para essas espécies. Tanto as pós-larvas quanto os juvenis não exibiram a interação típica entre os genes das alças positiva (*arntl* e *clock*)

e negativa (*per* e *cry*) e somente em pós-larvas a expressão gênica das enzimas digestivas é, provavelmente, influenciada pelos genes relógio.

Palavras-chave: *Aloysia thiphylla*. Anestesia. Ciclo circadiano. *Lippia alba*. mRNA. Ritmo alimentar

ABSTRACT

ESSENTIAL OILS AS ANESTHETICS IN SERRASALMDS AND GENE EXPRESSION OF CLOCK GENES AND DIGESTIVE ENZYMES IN SILVER CATFISH

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Date and Place of Defense: Santa Maria, August 31st, 2018.

Anesthetics of synthetic origin are widely used in aquaculture to minimize animal stress arising from the practices involved in this activity. However, the adverse effects caused by these synthetic substances have encouraged research for natural substances with sedative and anesthetics properties that are more effective, safer and with lower risk of adverse effects. Studies have shown that essential oils (EOs) extracted from the plants species *Aloysia triphylla* and *Lippia alba*, as well as eugenol, an isolated compound of clove oil, present sedative and anesthetic properties in fish, being considered promising alternatives to traditional anesthetics. In this context, the first part of this study aimed to investigate the effects of these EOs on two Amazonian species, *Serrasalmus rhombeus* and *S. eigenmanni*, inhabitantsof Rio Negro. In the second part, we evaluated the gene expression of clock and digestive enzymes genes in post-larvae and juvenile of *Rhamdia quelen*. In the article 1, the sedative and anesthetic efficacy of *A. triphylla* and *L. alba* EOs and eugenol and the recovery time was determined in *Serrasalmus rhombeus*, as well as the swimming behavior on a short exposure of 15 min at low concentrations to evaluate the possible use of these substances in the transport of this species. Both EOs and eugenol are effective, with concentrations of 150, 200, and 50 µL L^{-1}, respectively, being recommended for anestesia. For sedation, it is recommended to use 50 µL L^{-1} for both EOs and 25 µL L^{-1} for eugenol. The concentrations of 5 and 10 µL L^{-1} are indicated for transport studies with these anesthetics. In manuscript 2, the sedative and anesthetic efficacy of *A. triphylla* and *L. alba* EOs in *Serrasalmus eigenmanni*, as well as recovery time of the fish were determined. The net ion fluxes, blood parameters, and swimming behavior at a prolonged exposure of 4 h were also evaluated. Both EOs were effective for anesthesia of *S. eigenmanni* and the concentration of 100 µL L^{-1} was recommended for both EOs. For sedation, the indicated concentrations are 25 and 50 µL L^{-1} of *A. triphylla* and *L. alba* EOs, respectively. For transport, the concentration of 5 µL L^{-1} of *A. triphylla* EO is recommended due to the lower ammonia excretion and maintenance of fish equilibrium. In post-larvae, genes of the positive loop (*artnl1a* and *clock*) and the negative loop (*per1,2,3* and *cry1,2*) presented differences in expression levels throughout the evaluated period, with acrophase occurring in the same schedule. In juveniles, only one positive loop gene (*arntl1*) and one negative loop gene (*cry2*) showed differences in expression levels in the evaluated period, with a difference of 1 h between the acrophases. The levels of gene expression of the digestive enzymes in post-larvae were maximal 4 h before feeding and at least 1 h after feeding. In the digestive tract of juveniles the levels of gene expression of the digestive enzymes did not differ in the evaluated period. In conclusion, both OEs are effective for sedation and anesthesia of *S. rhombeus* and *S. eigenmanni*, and may also be used in transport studies for these species. Both post-larvae and juveniles did not exhibit the typical interaction between the genes of the positive (*arntl* and *clock*) and negative (*per* and *cry*) loops and only in post-larvae the gene expression of the digestive enzymes is probably influenced by the genes clock.

Keywords: *Aloysia thiphylla*. Anesthesia. Circadian cycle. Feeding. *Lippia alba*. mRNA.

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LISTA DE ABREVIATURAS

actb	Gene codificante para β-actina
amy2a	Gene codificante para α-amilase pancreática
ACTH	Hormônio adrenocorticotrófico
arntl	Gene codificante para aryl hydrocarbon receptor nuclear translocator-like
bmal	Gene codificante para brain and muscle ARNT-like protein
cDNA	DNA complementar
cel	Gene codificante para carboxil éster lipase ou lipase ativada por sais biliares
clock	Gene codificante para circadian locomotor output cycles kaput
CRH	Hormônio liberador de corticotrofina
cry	Gene codificante para cryptochrome
ctr2	Gene codificante para quimotripsinogênio 2
DNA	Ácido desoxirribonucleico
ef1a	Gene codificante para elongation factor 1ª
FAA	Food antecipatory activity
FEO	Food entrainable oscillator
GIT	Gastrointestinal tract
HHI	Eixo hipotalâmico-hipófise-interrenal
LD	Fotoperíodo claro/escuro
LEO	Light entrainable oscillator
LL	Fotoperíodo claro constante
mRNA	RNA mensageiro
NCBI	National Center for Biotechnology Information
NSQ	Núcleo supraquiasmático
OE	Óleo essencial
PCR	Reação em cadeia da polimerase
per	Gene codificante para period
pga2	Gene codificante para pepsinogênio 2a
pla2g1b	Gene codificante para fosfolipase 2a
qPCR	PCR quantitativo (PCR a tempo real)
RACE	Rapid amplification of cDNA ends
RNA	Ácido ribonucleico
try2	Gene codificante para tripsinogênio 2

SUMÁRIO

1 INTRODUÇÃO

1.1 ESTRESSE E ANESTESIA EM PEIXES

Os peixes enfrentam na natureza desafios como fuga de predador, disputa por alimento, disputa e/ou defesa de território, variações de pH, temperatura, turbidez, presença de substâncias tóxicas, patógenos, entre outros. Essas ameaças fazem com que o peixe entre em um estado diferente da homeostase, o qual podemos denominar estresse (BARTON e IWAMA, 1991; PORTZ, WOODLEY e CECH JR, 2006; BAKER, GOBUSH e VYNNE, 2013). Nesse estado, uma sequência de eventos fisiológicos é desencadeada com a finalidade de auxiliar o animal a reagir e, se possível, se recuperar.

A resposta ao estresse passa por três fases distintas denominadas Síndrome Geral da Adaptação, que consiste em uma fase inicial de alerta com uma rápida resposta fisiológica, seguida de uma segunda fase de resistência, que é quando o peixe tenta se adaptar a fim de recuperar a homeostase. Caso a adaptação não seja possível devido à duração/intensidade do estresse, o peixe entra em exaustão, a denominada terceira fase na qual a saúde e até mesmo a vida do animal é comprometida (BARTON e IWAMA, 1991; GERWICK et al., 1999; TORT, 2011). A resposta imediata ao estresse consiste na ativação simpática do eixo hipotalâmico-hipófise-interrenal (HHI), que desencadeará alterações fisiológicas classificadas em resposta primária, secundária e terciária (Figura 1). A resposta primária, também denominada neuroendócrina, consiste na estimulação nervosa do tecido cromafim, que libera imediatamente catecolaminas (adrenalina e noradrenalina) na corrente sanguínea. De forma complementar, através da estimulação do HHI, o hipotálamo secreta o hormônio liberador de corticotropina (CRH) que age na hipófise promovendo a liberação do ACTH, que atua no tecido interrenal modulando a síntese e liberação de cortisol no sangue (REID et al., 1998; ELLIS et al., 2011; COWAN, AZPELETA e LÓPEZ-OLMEDA, 2017). O estresse também pode elevar os níveis dos hormônios tiroxina, prolactina e somatolactina e suprimir os hormônios reprodutivos (BARTON, 2002). Na resposta secundária ocorrem alterações metabólicas afim de aumentar a produção de energia. O aumento da liberação de cortisol e catecolaminas na corrente sanguínea desencadeará alterações nas reservas de glicogênio hepático e muscular, na glicemia e no ácido lático, bem como alterações dos batimentos cardíacos e no balanço hidromineral. A adrenalina liberada na corrente sanguínea altera o fluxo sanguíneo nas brânquias, bem como a sua permeabilidade, resultando em uma disfunção hidromineral (SCHRECK e TORT, 2016). A exposição ao estresse crônico e a exaustão fisiológica, desencadearão a resposta terciária, a qual

interfere negativamente no desenvolvimento do animal através de mudanças na taxa de crescimento, reprodução, sobrevivência e imunidade (MAZEAUD et al., 1977; ELLIS et al., 2011; WEBER, 2011a).

Na aquicultura, os animais são submetidos a práticas estressantes diariamente, como manuseio, captura, biometria, análises patológicas, implantes hormonais, bem como o transporte, que pode expor os animais a altas densidades de estocagem, variações em parâmetros da água como temperatura, pH e oxigênio dissolvido, bem como exposição a barulho excessivo (BARTON, 2000; URBINATI et al., 2004; OLIVEIRA et al., 2009; BOLASINA, 2011; CUNHA et al., 2011; GOMES et al., 2011; BECKER et al., 2012). Com a finalidade de minimizar os efeitos do estresse e evitar que tais práticas interfiram na homeostase do animal afetando negativamente sua sobrevivência, substâncias com efeitos sedativos e anestésicos têm sido empregadas (COOKE et al., 2004; INOUE et al., 2005; BECKER et al., 2012).

A anestesia é um procedimento que age através da depressão generalizada do sistema nervoso central. É um processo reversível que pode ser causado por um fármaco e resulta na perda de percepção de parte ou de todo corpo (SUMMERFELT e SMITH, 2000). O procedimento anestésico pode ser dividido em fases principais: indução, manutenção e recuperação. Na indução o peixe é exposto ao agente anestésico, o que pode levar a uma agitação inicial passageira. Essa fase deve ser rápida e sem grande agitação do peixe. A fase de manutenção começa quando determinado estágio de anestesia é alcançado e existe a necessidade de manter o peixe nesse estado por um tempo. No entanto, considerando a concentração (ou dose, se for injetado) e o tempo de exposição, o anestésico pode se acumular no cérebro e no músculo, o que pode levar a uma exposição excessiva, prejudicando a saúde do animal. Na fase de recuperação, o anestésico é retirado e o peixe retorna ao estado normal, retomando a natação regular e a reação a estímulos externos. Dependendo do anestésico administrado, a recuperação total pode demorar minutos ou horas (COYLE, DURBOROW e TIDWELL, 2004).

Os anestésicos consistem em agentes químicos que inicialmente provocam um efeito calmante seguido de perda do equilíbrio, de mobilidade, de consciência e da ação reflexa. Os anestésicos podem agir de três modos: estabilizando os impulsos nervosos em axônios aferentes e/ou eferentes, bloqueando competitivamente os sítios receptores na membrana pós-sináptica e bloqueando a liberação de neurotransmissores na membrana pré-sináptica. O método de administração de anestésicos mais utilizado em peixes é o de banhos de imersão. A solução

anestésica presente na água é absorvida pelas brânquias difundindo-se através da corrente sanguínea até o sistema nervoso central (ROSS e ROSS, 2008).

Figura 1 – Respostas primária, secundária e terciárias ao estresse em peixes.
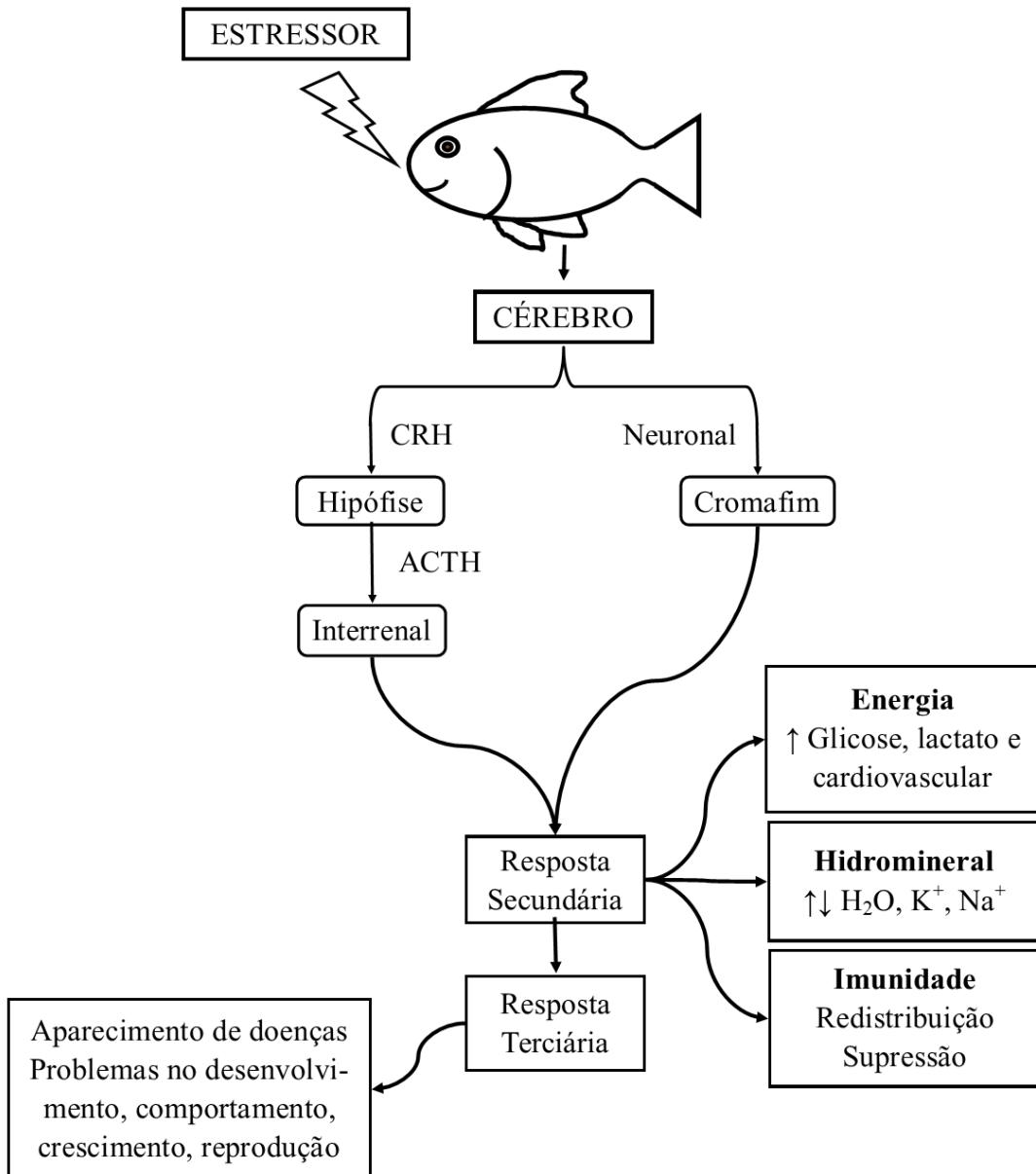
Fonte: Adaptado de Schreck e Tort (2016). CRH – hormônio liberador de corticotropina, ACTH – hormônio adrenocorticotrópico.

A maioria dos agentes anestésicos pode produzir vários estágios de anestesia. Small (2003) descreve quatro estágios para anestesia: Estágio I – Sedação, com a diminuição na reatividade a estímulos externos; Estágio II – Ocorre perda parcial do equilíbrio e o peixe apresenta natação errática; Estágio III – Ocorre perda total do equilíbrio e a locomoção cessa;

Estágio IV – Colapso medular e morte. A recuperação do peixe é definida pela retomada da natação regular e da reação a estímulos externos.

O anestésico ideal é descrito como uma substância que seja eficaz em baixas concentrações, induzindo rapidamente a sedação e a anestesia no animal com mínima hiperatividade do mesmo. Além disso, deve ser de fácil manuseio, baixo custo, disponibilidade alta e ser seguro tanto para os animais a serem expostos quanto para quem o manipula, bem como para o meio ambiente (ROSS e ROSS, 2008). Os anestésicos de origem sintética mais empregados na anestesia de peixes são o metanosulfonato de tricaína (MS222), o metomidato, a benzocaína, a quinaldina, e o 2-fenoxietanol (ROSS e ROSS, 2008; MARICCHIOLO e GENOVESE, 2011). Ainda não há na legislação brasileira a regulamentação do uso de anestésicos na aquicultura, logo procura-se adotar as regras definidas pela Food and Drug Administration (FDA) para a utilização de substâncias anestésicas em peixes. A FDA recomenda a utilização do MS-222 em anestesia de peixes, todavia esse produto necessita ser importado, onerando a sua utilização (FAÇANHA e GOMES, 2005). Ademais, a exposição do peixe a esses compostos sintéticos pode causar efeitos adversos como perda de muco, acidose, alterações nos níveis plasmáticos de lactato, ureia, hormônios, colesterol, glicose e eletrólitos, imunossupressão, hipóxia tecidual e aumento do hematócrito (CARTER, WOODLEY e BROWN, 2011; GOMES et al., 2011; ZAHL et al., 2011; SNEDDON, 2012). Diante disso, alternativas naturais, tais como os óleos essenciais provenientes de plantas, têm sido exploradas com a finalidade de substituir substâncias sintéticas.

Os óleos essenciais (OEs) são extratos vegetais compostos de metabólitos secundários voláteis (CARSON et al., 2006) que à temperatura ambiente são líquidos de coloração que varia do incolor ao amarelo, solúveis em solventes orgânicos e instáveis na presença de calor, umidade, luz e metais (RAUT e KARUPPAYIL, 2014). São sintetizados por plantas aromáticas, logo são caracterizados por intenso odor. Apresentam a importante função de proteger as plantas, atuando como agentes antibacterianos, antifúngicos, inseticidas e contra herbívoros, desencorajando a sua ingestão. No entanto, podem agir como atrativos para alguns insetos, auxiliando a polinização e a dispersão de sementes (BAKKALI et al., 2008). Esses extrativos vegetais apresentam vasta aplicação na produção de perfumes, cosméticos, produtos farmacêuticos, alimentícios e de higiene, por apresentarem caráter terapêutico, como flavorizantes e aromatizantes (SPEZIALI, 2012). Estudos demonstraram uma ampla gama de atividades biológicas dos OEs: antimicrobiana, virucida, antiparasitária, antioxidante, cardiovascular, antidiabética, sedativa e anestésica (BAKKALI et al., 2008).

Estudos revelam resultados promissores do uso de OEs e seus constituintes como anestésicos em peixes. Os OEs de cravo (*Eugenia aromatica*) (ROUBACH et al., 2005; CUNHA et al., 2010; INOUE et al., 2011; MOREIRA et al, 2010; WEBER et al., 2011b), *Ocimum gratissimum* (BENOVIT et al., 2012), *Hesperozygis ringens* (TONI et al., 2013; 2015a), *Ocotea acutifolia* (SILVA et al., 2013), *Melaleuca alternifolia* (HAJEK, 2011), *Nectandra megapotamica* (TONDOLO et al., 2013), *Mentha piperita* (MAZANDARANI e HOSEINI, 2018), *Aloysia polystachya* (FOGLIARINI et al., 2017), *Nectandra grandiflora* (BARBAS et al., 2017), *Myrcia sylvatica* e *Curcuma longa* (SACCOL et al., 2017) demonstraram eficácia anestésica em diversas espécies de peixes. Compostos isolados de plantas como mentol (MAZANDARANI e HOSEINI, 2017), linalol (HELDWEIN et al., 2014), mirceno (MIRGHAED et al., 2018), timol e carvacrol (BIANCHINI et al., 2017) também demonstraram eficácia nas espécies testadas.

Na família Verbenaceae, podemos destacar as espécies *Aloysia triphylla* (L´Hér.) Britton e *Lippia alba* (Mill.) N. E. Brown. A espécie *A. triphylla* é popularmente conhecida como cidró, cidrão e limonete. É nativa da América do Sul e cultivada no sul do Brasil, apresentando porte arbustivo, ramificado e ereto, podendo alcançar até 3 m de altura. Possui propriedades adstringente e aromática, rica em óleo volátil que age como sedativo brando (LORENZI e MATOS, 2008). Suas folhas apresentam grande importância medicinal, uma vez que o chá das folhas apresenta propriedades antipirética, analgésica e anti-inflamatória, bem como propriedades sedativas, antioxidantes e digestivas (EL-HAWARY et al., 2012). Também é utilizada na culinária, empregada como aromatizante de ambientes, bactericida e inseticida (LORENZI e MATOS, 2008). Estudos com OE de *A. triphylla* demonstraram sua eficácia anestésica em *Rhamdia quelen* (CUNHA et al., 2010; GRESSLER et al., 2014; PARODI et al., 2014; ZEPPENFELD et al., 2014; BECKER et al., 2015), *Oreochromis niloticus* (TEIXEIRA et al., 2017) e *Litopennaeus vannamei* (PARODI et al., 2012). A espécie *L. alba* é popularmente conhecida como erva cidreira de arbusto, sendo, também, nativa da América do Sul (BIASI e COSTA, 2003). Apresenta porte arbustivo muito ramificado, as brotações tendem a arquear conforme o crescimento, encostando-se ao solo e enraizando formando moita de até 2 m de altura (SILVA JUNIOR, 1998). Possui propriedades analgésica, anti-inflamatória, antipirética, antidiarreica, antiespasmódica e sedativa. Utilizada também no tratamento de doenças do trato respiratório e como tempero na culinária (PASCUAL et al., 2001). O OE de *L. alba* já teve sua eficácia anestésica comprovada em *Hippocampus reidi* (CUNHA et al., 2011), *R. quelen* (CUNHA et al, 2010; HELDWEIN et al., 2014; TONI et al., 2013), *L. vannamei* (PARODI et al, 2012), *Sparus aurata* (TONI et al., 2015b) e *Argyrosomus regius* (CÁRDENAS et al., 2016).

Na família Myrtaceae, podemos destacar a espécie *Eugenia aromatica*, popularmente conhecida como cravo da índia. O eugenol é o principal componente do óleo essencial de cravo e é tradicionalmente utilizado na odontologia devido a suas propriedades analgésica e antisséptica (CHAIEB et al., 2007). Estudo demonstrou a eficácia da utilização desse composto isolado para anestesia em peixes (CUNHA et al., 2010).

Figura 2 – *Aloysia triphylla*.   Figura 3 – *Lippia alba*.   Figura 4 – Eugenol

Fonte: http://jb.utad.pt/especie/aloysia_triphylla   Fonte: Hennebelle et al., 2008.   Fonte: www.maquira.com.br

1.2 RITMOS BIOLÓGICOS EM PEIXES

Os ritmos biológicos são definidos como todo evento que repete regularmente em um organismo. Os ritmos mais explorados são os relacionados às variações ambientais cíclicas, as quais permitem ao organismo se adaptar. No caso de variações ambientais acíclicas, a imprevisibilidade interfere na expressão rítmica dos ritmos biológicos (MORGAN, 2004). Considerando que, praticamente, todos os ambientes naturais apresentam peculiaridades cíclicas, os organismos também precisam oscilar para se adaptar e sincronizar suas atividades biológicas aos ciclos ambientais. Evolutivamente, os organismos desenvolveram estratégias comportamentais e fisiológicas que os permitem antecipar variações previsíveis no ambiente e, consequentemente, adaptar suas atividades às variações ambientais, otimizando seus processos biológicos (MARQUES e MENNA-BARRETO, 2003; VERA et al., 2009).

Uma propriedade básica dos ritmos biológicos endógenos é que eles devem se manter em condições ambientais constantes, isto é, surgem no próprio organismo e não por influência externa (CYMBOROWSKI, 2010). O ritmo circadiano apresenta período de aproximadamente 24 horas, podendo ser ajustado através da mudança gradativa de fase de oscilação até se

equiparar ao ciclo ambiental e estabilizar (arrastar). O arrastamento é produzido por sinais externos, conhecidos como *zeitgebers* ou sincronizadores, como os estímulos ambientais (ZHDANOVA e REEBS, 2006). O sincronizador mais poderoso parece ser a luz, pois os organismos tendem a sincronizarem seus ciclos atividade/repouso com a alternância diária do ciclo claro/escuro ambiental (VERA et al., 2007). Contudo, existem outros parâmetros ambientais críticos que apresentam uma variação diária ou anual, por exemplo o risco de predação, temperatura ambiental, hierarquia social e disponibilidade de alimento que podem atuar como sincronizadores e, dependendo da espécie, exercer um papel mais importante que a luz (ZHDANOVA e REEBS, 2006).

Basicamente, o sistema circadiano inclui receptores que captam a informação do ambiente e transmitem a um oscilador central localizado no cérebro que sincroniza e transmite a mensagem até os osciladores periféricos, localizados em outros órgãos. Todavia, dependendo do organismo em questão, a informação ambiental pode agir diretamente em determinado oscilador periférico sem precisar passar pelo oscilador central (CYMBOROWSKI, 2010).

Nos mamíferos, o oscilador central que define a maioria dos ritmos circadianos é o núcleo supraquiasmático (NSQ). A informação fótica é percebida por células fotossensíveis presentes na retina e é transmitida através do trato retino-hipotalâmico até o NSQ, que está localizado no sistema nervoso central, ativando a transcrição de genes relógios e sincronizando a atividade neuronal. Os sinais enviados pelo NSQ controlam, por exemplo, a síntese noturna da melatonina pela glândula pineal (MAYWOOD et al, 2007; DIBNER, SCHIBLER e ALBRECHT, 2010). Em peixes, devido à ausência de uma estrutura homóloga ao NSQ de mamíferos, a glândula pineal apresenta grande importância na ritmicidade circadiana. Essa glândula é fotossensível e possui relógio intrínseco responsável pela produção e liberação da melatonina (FÁLCON et al., 2010) e pela expressão de genes relógio (NOCHE et al., 2010).

A nível molecular, o funcionamento do relógio biológico está baseado na interação entre alças de retroalimentação transcricional-translacional positivas e negativas de genes relógio e o mesmo é semelhante em todos os seres vivos que apresentam um relógio circadiano (Figura 5). Os genes relógio são encontrados em várias regiões do sistema nervoso central e em vários tecidos periféricos (CYMBOROWSKI, 2010).

A alça positiva é composta pelos genes *circadian locomotor output cycles kaput* (*clock*) e *brain and muscle ARNT-like protein* (*bmal* ou *arntl*), que são transcritos no núcleo e traduzidos em proteínas no citoplasma e interagem através do domínio PAS (*Period-ARNT-SINGLE-Minded*), presentes nessas proteínas, formando um heterodímero que retorna ao núcleo. No núcleo, o domínio bHLH (*basic Helix-Loop-Helix*) permite que o heterodímero

clock/bmal se ligue aos elementos *E-box* dos genes da alça negativa, induzindo sua transcrição. A alça negativa é formada pelos genes *period* (*per1*, *per2* e *per3*) e *cryptochrome* (*cry1* e *cry2*), que são transcritos no núcleo e traduzidos em proteínas no citoplasma que interagem formando um heterodímero *per/cry* que retorna ao núcleo e, quando presente em níveis elevados, interagem fisicamente, inibindo a transcrição mediada pelo complexo *clock/bmal*. Simultaneamente, o complexo *clock/bmal* inicia uma alça auxiliar de retroalimentação para ativar a transcrição dos genes *rorα* (*ROR-related orphan receptor α*) e *rev-erbα* que são traduzidos no citoplasma e suas proteínas (RORα e REV-ERBα, respectivamente) competem pela ligação em RORE (*Retinoic Acid-Related Orphan Receptor Reponse Element*) localizado na região promotora do gene *bmal*. As proteínas desses genes executam ações opostas em *bmal*: enquanto RORα ativa a transcrição, REV-ERBα a inibe. Assim, níveis elevados de *bmal* inibem sua transcrição através da elevação dos níveis dos genes *rev-erbα*, *per* e *cry*, enquanto que a ausência das proteínas desses genes ativa a transcrição de *bmal*, o qual irá formar novamente o complexo *clock/bmal* reiniciando o ciclo (ROSBASH, 1995; PEREIRA, TUFIK e PEDRAZZOLI, 2008; CECCON e FLÔRES, 2010; VATINE et al., 2011).

Figura 5 – Representação básica do funcionamento do relógio molecular.
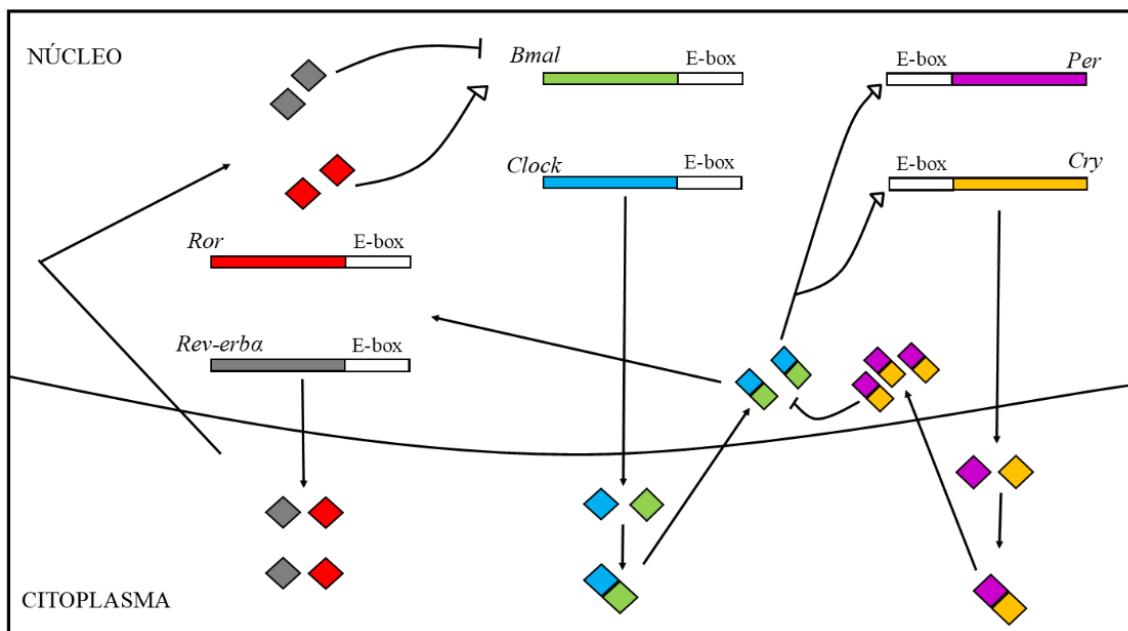
Fonte: Autor

1.2.1 Ritmo alimentar em peixes

Os ciclos claro/escuro e alimentar são considerados os *zeitgebers* mais fortes dos ritmos biológicos em animais. Na natureza não há uma oferta continua de alimento, mas restrita quanto ao local e ao horário (LÓPEZ-OLMEDA e SÁNCHEZ-VÁZQUEZ, 2010). A maioria dos animais, quando expostos à disponibilidade periódica de alimento, apresenta um aumento da atividade locomotora momentos antes do fornecimento do alimento, comportamento conhecido como atividade alimentar antecipatória (ou *food anticipatory activity* – FAA). A aquisição de alimentos, frequentemente, está vinculada ao ciclo claro/escuro devido à dependência da visão para a captura das presas (MADRID et al., 2010).

Em estudos com mamíferos pressupõe-se a existência de dois osciladores: um arrastado pela luz (*light entrainable oscillator* – LEO), localizado no NSQ do hipotálamo; e outro arrastado pelo alimento (*food entrainable oscillator* – FEO), cuja localização anatômica é incerta. Em mamíferos foi demonstrada a existência de um FEO independente do LEO, pois lesões no SNC não abolem a FAA (CYMBOROWSKI, 2010). Em peixes, não podemos descartar a influência do LEO sob o FEO e o grau de influência entre eles pode variar entre as espécies, conforme os padrões de atividade e hábitos alimentares. A luz será o sincronizador mais forte para uma espécie diurna que utiliza a visão para a captura de presas. Contudo, a disponibilidade de alimento exercerá influência maior em uma espécie noturna que utiliza, por exemplo, barbilhões para detectar o alimento (BOLLIET, ARANDA e BOUJARD, 2001).

A influência do ciclo claro/escuro (LD) e do horário de alimentação na expressão dos genes relógio no cérebro (oscilador central) e em órgãos periféricos de peixes já foi estudada em várias espécies. Estudo com dois grupos de robalos (*Dicentrarchus labrax*) submetidos a um fotoperíodo LD 12:12 h (light:dark), um alimentado na fase diurna e outro na fase noturna, demonstrou que tanto a luz quanto o alimento influenciaram a expressão gênica de *per1* no fígado (DEL POZO et al., 2012). Semelhante ao robalo, o ritmo de expressão de *per1* no fígado de zebrafish (*Danio rerio*) alimentados em diferentes horários foi alterado pelo horário de alimentação (SÁNCHEZ-VÁZQUEZ, ZAMORA e MADRID, 1995; SÁNCHEZ e SÁNCHEZ-VÁZQUEZ, 2009). Por outro lado, a expressão de genes relógio em dourada (*Sparus aurata*) é influenciada somente pelo alimento e o horário de alimentação pode mudar a fase do ritmo diário da expressão desses genes (VERA et al., 2013). Em robalo, o FEO e o LEO parecem estar acoplados, no entanto em dourada parece ocorrer um desacoplamento. No caso do peixinho dourado (*Carassius auratus*) foi demonstrado que os ritmos da expressão dos genes relógio *per3* e *cry3* no fígado dessa espécie, quando exposta a fotoperíodo LL, estão em

fase com a expressão desses genes no fígado e intestino dessa espécie quando exposta a um fotoperíodo 12:12 LD e alimentados diariamente no mesmo horário. Sugerindo que, nessa espécie, o horário de alimentação é um forte sincronizador desses genes mesmo quando submetidos a um ciclo LD (VELARDE et al., 2009; FELICIANO et al., 2011). Quanto à expressão gênica no cérebro, nos estudos acima citados somente o ciclo LD afetou a expressão dos genes relógio no oscilador central (cérebro) enquanto que os osciladores periféricos (fígado e intestino) podem ser afetados tanto pela luz e pelo alimento como somente pelo alimento.

As enzimas digestivas também podem apresentar uma ritmicidade na sua atividade demostrando que o ritmo circadiano pode exercer um importante controle funcional no sistema digestório (SCHEVING et al., 2000). Mata-Sotres et al. (2016) demonstrou que tanto a atividade quanto a expressão gênica das enzimas digestivas apresentam variações diárias dependentes do ciclo LD em larvas de *S. aurata* durante a ontogenia. Também demostrou uma tendência ao aumento na expressão gênica das enzimas pancreáticas no período escuro, o qual pode estar associado a uma preparação para a próxima ingestão de alimentos, uma vez que essas larvas foram alimentadas diariamente no período claro. A enzima amilase demonstrou um ritmo diário em *Semaprochilodus taeniurus*, com maiores níveis de atividade quando o estômago dos animais estava vazio como uma preparação para a próxima ingestão de alimento (LÓPEZ-VÁSQUEZ, CASTRO-PÉREZ e VAL, 2000). A atividade dessa mesma enzima apresentou sincronização com o regime alimentar em *C. auratus* alimentados diariamente no mesmo horário (VERA et al., 2007).

1.3 MODELOS EXPERIMENTAIS UTILIZADOS NO ESTUDO

1.3.1 *Serrasalmus rhombeus* (Linnaeus, 1766)

Espécie amazônica pertencente à ordem Characiformes e família Serrasalmidae. (JEGU E INGENITO, 2007). É uma espécie onívora, com preferência por peixes pequenos, invertebrados e plantas (SANTOS et al., 2006).

Figura 6 – *Serrasalmus rhombeus*

Fonte: I. Mikolji, retirada do FishBase.

1.3.2 *Serrasalmus eigenmanni* Norman, 1929

Espécie amazônica pertencente à ordem Characiformes e família Serrasalmidae, com hábito alimentar carnívoro (POUILLY et al., 2003).

Figura 7 – *Serrasalmus eigenmanni*

Fonte: Arquivo pessoal

1.3.3 *Rhamdia quelen* (Quoy E Gaimard, 1824)

Espécie pertencente à ordem Siluriformes e família Heptapteridae. Distribui-se em praticamente todas as bacias hidrográficas brasileiras (BOCKMANN, 2007). Espécie bentônica, noturna e com hábito alimentar onívoro (GOMES et al., 2000).

Figura 6 – *Rhamdia quelen*

Fonte: Alexssandro Geferson Becker.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os efeitos dos óleos essenciais de *Aloysia triphylla* e *Lippia alba* em *Serrasalmus rhombeus* (Linnaeus, 1766) e *Serrasalmus eigenmani* Norman, 1929 e a expressão gênica de enzimas digestivas e de genes relógios em *Rhamdia quelen* (Quoy E Gaimard, 1824).

2.2 OBJETIVOS ESPECÍFICOS

- Determinar a concentração ideal em cada óleo essencial e de eugenol para anestesia de *S. rhombeus* e *S. eigenmanni*.

- Investigar o tempo de indução e recuperação anestésica em *S. rhombeus* e *S. eigenmanni*.

- Analisar o comportamento natatório de *S. rhombeus* durante curta exposição aos anestésicos.

- Analisar o comportamento natatório e fluxo iônico corporal durante a exposição prolongada aos óleos essenciais em *S. eigenmanni*.

- Avaliar a expressão gênica das enzimas digestivas e de genes relógios em *R. quelen* em período de 24 h.

3 DESENVOLVIMENTO

3.1 ARTIGO 1

Essential oils and eugenol as anesthetics for *Serrasalmus rhombeus*

ESSENTIAL OILS AND EUGENOL AS ANESTHETICS FOR
*Serrasalmus rhombeus**

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ABSTRACT

This study evaluated the periods of time of anesthetic induction and recovery of *Serrasalmus rhombeus* exposed to essential oils (EOs) of *Aloysia triphylla* and *Lippia alba* and eugenol, as well as if these anesthetics can be used for transport of this species through analysis of swimming behavior. Fish were placed in aquaria containing different concentrations of *A. triphylla* EO or *L. alba* EO or eugenol, posteriorly were transferred to aquaria containing only water to evaluate the recovery time. In the second experiment, behavior was analyzed during exposure to *A. triphylla* EO, *L. alba* EO or eugenol at 5 or 10 µL L⁻¹. The evaluations were carried out at 0, 1, 5, 10 and 15 min of exposure. Fish exposed to 150, 200 and 50 µL L⁻¹ of *A. triphylla* EO, *L. alba* EO and eugenol, respectively, showed anesthetic induction time lower than 3 min and recovery time lower than 10 min. Concentrations of 50 µL L⁻¹ of both EOs and 25 µL L⁻¹ eugenol caused only sedation. Exposure to 5 and 10 µL L⁻¹ EOs and eugenol decreased fish swimming time. Both EOs and eugenol were effective for anesthesia and can be used for transport of *S. rhombeus*.

Key words: anesthesia; behavior; black piranha; fish transport; Negro river.

ÓLEOS ESSENCIAIS E EUGENOL COMO ANESTÉSICO PARA
Serrasalmus rhombeus

RESUMO

Esse estudo avaliou os períodos de tempo de indução e recuperação anestésica de *Serrasalmus rhombeus* expostos aos óleos essenciais (OEs) de *Aloysia triphylla* e *Lippia alba* e eugenol, bem como se esses anestésicos podem ser usados no transporte dessa espécie através da análise do comportamento natatório. Os peixes foram colocados em aquários contendo diferentes concentrações de OE de *A. triphylla* ou OE *L. alba* ou eugenol, posteriormente foram transferidos para aquários contendo somente água para avaliar o tempo de recuperação. No segundo experimento, comportamento foi analisado durante exposição aos OEs ou eugenol nas concentrações 5 ou 10 µL L⁻¹. As avaliações foram realizadas em 0, 1, 5, 10 e 15 min de exposição. Peixes expostos a 150, 200 e 50 µL L⁻¹ de OE de *A. triphylla*, OE de *L. alba* e eugenol, respectivamente, apresentaram tempo de indução anestésica menor que 3 min e tempo de recuperação menor que 10 min. Concentrações de 50 µL L⁻¹ de ambos OEs e 25 µL L⁻¹ de eugenol causaram somente sedação. Exposição a 5 e 10 µL L⁻¹ de OEs e eugenol diminuíram o tempo de natação dos peixes. Ambos os OEs e o eugenol foram efetivos para anestesia e podem ser utilizados para transporte de *S. rhombeus*.

Palavras-chave: anestesia; comportamento; piranha preta; transporte de peixes; rio Negro.

INTRODUCTION

There is a growing interest in the search for natural anesthetics, obtained from plants, which are economically viable and present low toxicity. The most widely used natural anesthetic is eugenol (4-allyl-2-methoxyphenol), the main compound from clove oil, which is obtained from plants of the genus *Eugenia* (ANDERSON *et al.*, 1997). The anesthetic efficacy of eugenol was showed for several Neotropical species as *Piaractus mesopotamicus* (Holmberg, 1887) (GONÇALVES *et al.*, 2008), *Astyanax bimaculatus* (Linnaeus, 1758) (SILVA *et al.*, 2009), *Brycon amazonicus* (Spix & Agassiz, 1829) (VIDAL *et al.*, 2007), *Rhamdia quelen* (Quoy & Gaimard, 1824) (CUNHA *et al.*, 2010a), *Carassius auratus* (Linnaeus, 1758) (BITTENCOURT *et al.*, 2012), *Centropomus parallelus* Poey, 1860 (SOUZA *et al.*, 2012) and *Brycon hilarii* (Valenciennes, 1850)

(FABIANI *et al.*, 2013). The efficacy of the essential oil (EO) of *Lippia alba* was also demonstrated for *Rhamdia quelen* (CUNHA *et al.*, 2010b; HELDWEIN *et al.*, 2012, 2014; TONI *et al.*, 2014), *Hippocampus reidi* (Ginsburg, 1933) (CUNHA *et al.*, 2011) and *Sparus aurata* (Linnaeus, 1758) (TONI *et al.*, 2015), but the EO of *Aloysia triphylla* was studied only in *R. quelen* (PARODI *et al.*, 2014) and *C. parallelus* (PARODI *et al.*, 2016).

Serrasalmus rhombeus (Linnaeus, 1766), popularly known as black piranha or redeye piranha, is distributed across Amazon and Orinoco basins, Guiana Shield rivers and coastal rivers of northeastern Brazil (JÉGU and INGENITO, 2007). This species is an important fisheries resource (MPA, 2011) and has been exported as ornamental fish (ANJOS *et al.*, 2009), but has powerful teeth that can cause serious injury in humans (MOL, 2006). Therefore, the use of anesthesia in the management of this fish is indicated to prevent injuries in the handlers and to reduce the effects of animal stress.

The aim of this study was to evaluate the anesthetic efficacy of EOs of *A. triphylla* and *L. alba* and eugenol in *S. rhombeus*. In addition, we also investigated the swimming behavior of *S. rhombeus* exposed to low concentrations of these anesthetics to indicate if they could be used in the water of transport of this species.

METHODS

Essential oils and eugenol

The plants *A. triphylla* and *L. alba* were cultivated at the campus of the Universidade Federal de Santa Maria in the city of Frederico Westphalen, southern Brazil. Voucher specimens (SMDB No. 11169 and 10050, respectively) were deposited in the herbarium of the Biology Department. Eugenol (99.9%, Maquira®) was purchased in a local drugstore.

Essential oil extraction and analysis

The oil extraction from the leaves of these plants was performed by hydro-distillation using Clevenger apparatus according to the European Pharmacopoeia (2007). Analysis was made by gas chromatography using an Agilent 7890A gas equipment coupled to an Agilent 5975C mass selective detector (GC-MS). The unit was equipped with a capillary column HP5-MS (Hewlett Packard, 5% fenilmetilsiloxane, 30 m x 0.25 mm, film thickness: 0.25 μm), and the ionization energy used was 70 eV. The parameters choose for the analysis were: He as gas carrier; split inlet 1:100; temperature program: 40°C for 4 minutes; 40 to 320°C at 4°C min⁻¹; 1 mL min⁻¹ of flow rate; and temperatures of injection and detection of 250°C. The chemical compounds identification was made by comparison of retention indexes, obtained by using a calibration curve of n-alkanes injected at the conditions mentioned for the samples, and the mass fragmentation patterns with NIST (2010) data.

Animals

Specimens of *S. rhombeus* (14.9 ± 0.51 cm, 110.9 ± 3.79 g, voucher number: INPA-ICT 53086) were collected during an expedition to Anavilhanas Islands of the Negro River, 110 km upstream from Manaus (2°23'41"S, 60°55'14"W). Fish were maintained in tanks with 50% water daily renewed, pumped directly from Rio Negro (29.8°C, pH 5.0), continuously aerated for a few hours before testing.

Experiment I: anesthesia induction and recovery in S. rhombeus exposed to A. triphylla and L. alba EOs and eugenol

The fish were transferred individually to aquaria containing 2 L (29.8 ± 0.46°C; pH 5.0 ± 0.1) of water with the *L. alba* (50, 100 and 200 μL L⁻¹) or *A. triphylla* EOs (50, 100 and 150 μL L⁻¹) or eugenol (25, 40 and 50 μL L⁻¹), first diluted in ethanol at a proportion of 1:10. These concentrations were chosen based in previous studies (CUNHA *et al.*, 2010a, b; PARODI *et al.*, 2014). A total of 14 compounds were identified in each essential oil. The main constituents of *A. triphylla* EO were limonene (21.69%) and geranial (24.32%) and of *L. alba* EO were linalool (66.35%) and eucalyptol (10.63%) (Table 1).

The time for anesthesia induction was evaluated according to SMALL (2003): stage I - corresponds to sedation, when the reactivity to external stimuli decreased; stage II – corresponds to partial loss of equilibrium and erratic swimming; and stage III – corresponds to total loss of equilibrium and cessation of locomotion. In recovery, the fish returns to regular swimming. Eight fish were used for each tested concentration and each fish was used only once. The maximum observation time was 15 min, since several studies indicated that sedation and anesthesia occur within this period (CÁRDENAS *et al.*, 2016; HOHLENWERGER *et al.*, 2016; PARODI *et al.*, 2016; SENA *et al.*, 2016; TEIXEIRA *et al.*, 2017). Control experiment was performed using aquaria containing water and ethanol at a concentration equivalent to the highest dilution (1800 μL L⁻¹). After induction of anesthesia, fish were transferred to a tank containing only water to evaluate the recovery time. The animals were recovered when swimming regularly and reacting to external stimuli (the peduncle of the caudal fin was pressed with a glass rod).

Experiment II: fish behavior through exposure to low concentrations of A. triphylla and L. alba EOs and eugenol

The fish were placed into tanks containing 20 L of water and *A. triphylla* or *L. alba* EOs or eugenol at 5 and 10 μL L⁻¹. These concentrations were 2.5 and 5-fold lower than the lowest eugenol concentration tested, which induced stage II (partial loss of equilibrium) (see results) and also based on studies carried out by BECKER *et al.* (2012, 2013) and PARODI *et al.* (2014). Four fish per aquaria were used for each concentration (in triplicate). The fish were filmed for 20 s for analysis of the total swimming time and equilibrium (partial or total loss of equilibrium or normal) at 0, 1, 5, 10 and 15 min of exposure. Control experiments were performed using aquaria containing only water and aquaria containing ethanol at a concentration equivalent to that used in the highest dilution of the EOs (90 μL L⁻¹).

Table 1. Chemical composition of essential oils.

Essential Oil	RI* Experimental	RI Literature[a]	Chemical Compound	Percent Composition
A. tryphilla	989	986	5-Hepten-2-one, 6-methyl	2.085
	991	986	β-Pinene	0.499
	1026	1026	Limonene	21.694
	1049	1048	β -cis-Ocimene	0.717
	1229	1228	cis-Geraniol	2.218
	1241	1247	cis-Carveol	18.533
	1256	1259	Linlyl Acetate	2.703
	1271	1269	Geranial	24.317
	1417	1415	β-Caryophyllene	5.323
	1483	1483	α-Curcumene	3.251
	1495	1487	(-)-Alloaromadendrene	1.136
	1577	1578	Spathulenol	2.617
	1582	1583	Caryophyllene Oxide	6.793
	1640	1639	T-Cadinol	1.411
			Identified compounds	**93.297**
L. alba	971	969	Sabinene	0.817
	992	996	β-Pinene	0.972
	1027	1026	Limonene	1.992
	1028	1030	Eucalyptol	10.633
	1100	1101	Linalool	66.347
	1143	1146	Camphor	0.516
	1204	1205	Trans-Dihydrocarvone	1.183
	1242	1252	Carveol	1.135
	1272	1270	Geranial	0.764
	1273	1270	Neral	0.361
	1418	1419	Aromadendrene	3.480
	1480	1482	Germacrene D	2.784
	1556	1558	Germacrene B	2.219
	1582	1582	Spathulenol	1.340
			Identified Compounds	**94.543**

*RI = Retention Index. [a]NIST, 2010.

Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances among treatments was tested by Levene's test. The data from time to induction of anesthetic stages presented homogeneous variances and comparisons between the different concentrations were assessed using one-way ANOVA and Tukey's test. The data from swimming time did not exhibit homogeneous variances; therefore, comparisons between the different treatments and times were assessed using the non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test followed by the post-hoc Nemenyi test. The analysis was performed using the Statistica 7.0 software (Stat Soft. Inc.) and the minimum significance level was set at $P<0.05$.

RESULTS

The concentrations of 50 µL L⁻¹ *A. triphylla* EO, 50 µL L⁻¹ *L. alba* EO and 25 µL L⁻¹ eugenol only induced sedative effect within 15 min, but all other assay concentrations induced all stages of anesthesia. Fish exposed to 150, 200 and 50 µL L⁻¹ of *A. triphylla* and *L. alba* EO and eugenol, respectively, reached deep anesthesia (stage 3) significantly faster than the lower concentrations (Figure 1). The increasing concentration of *L. alba* EO increased proportionally recovery time (Figure 1B). The *A. triphylla* EO increased recovery time up to 100 µL L⁻¹ and eugenol only at the highest concentration (50 µL L⁻¹) (Figures 1A, C). The fish showed at the first contact with the anesthetics in both tests small jumps and swimming bursts in the first experiment. The application of 1800 µL L⁻¹ ethanol alone did not produce any anesthetic effect.
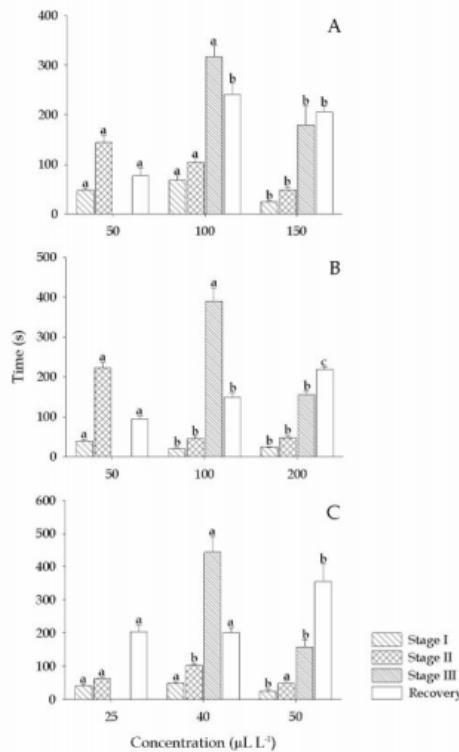
Figure 1. Time required for induction and recovery of anesthesia in *Serrasalmus rhombeus* using *Aloysia triphylla* (A) and *Lippia alba* (B) essential oils and eugenol (C). Different letters indicate a significant difference in the same stage based on one-way ANOVA and Tukey's test (P < 0.05).

Figure 2. Swimming time of *Serrasalmus rhombeus* exposed to low concentrations of *Aloysia triphylla* (A) and *Lippia alba* (B) essential oils and eugenol (C). Different letters indicate significant difference between times of exposure. *indicates significant difference from control fish. # indicates significant difference from ethanol-exposed fish (P < 0.05).

In the second experiment, fish exposed to 10 μL L⁻¹ EO of *A. triphylla*, 5 and 10 μL L⁻¹ EO of *L. alba* and 5 μL L⁻¹ of eugenol presented lower swimming time than control fish in all evaluated times. The fish did not show loss of equilibrium at both concentrations of EOs and eugenol tested. Ethanol-exposed fish initially decreased swimming time, but returned to the control level after 10 min (Figure 2).

DISCUSSION

Aloysia triphylla EO used in this study presented in its chemical composition limonene and geranial as main constituents. Unlike the present study, other studies detected citral, which is composed of geranial and neral, as main constituents of this EO (FIGUEIREDO *et al.*, 2004;

SARTORATTO *et al.*, 2004; PAULUS *et al.*, 2013). *Lippia alba* EO presented as the main constituent linalool, as observed in previous studies (HELDWEIN *et al.*, 2012; TONI *et al.*, 2015).

The anesthetic and sedative effects of eugenol are well established in rodents and fish (CUNHA *et al.*, 2010a; FREIRE *et al.*, 2006). On the other hand, the central depressor effects detected for the EOs of *A. triphylla* and *L. alba* are due to the association of different components, and resulted from additive and/or synergistic activities. For some of their constituents, as linalool and spathulenol, anesthetic and sedative effects were already described in *Rhamdia quelen* (BENOVIT *et al.*, 2015; HELDWEIN *et al.*, 2014). Additionally, some components detected in the EOs showed sedative and/ or anxiolytic like properties in mice, as limonene, geranial, 1,8-cineole and ß-pinene

(GOMES *et al.*, 2010; GUZMÁN-GUTIÉRREZ *et al.*, 2012; VALE *et al.*, 2002).

The present study demonstrates that eugenol and the EOs of *A. triphylla* and *L. alba* presented anesthetic effect in *S. rhombeus*. According to PARK *et al.* (2009), the suitable anesthetic induction time is about 3 min and at most 10 minutes for recovery. Following this premise, the best concentrations of the EOs of *A. triphylla* and *L. alba* and eugenol to anesthetize *S. rhombeus* were 150, 200 and 50 µL L^{-1}, respectively. The time required to induce deep anesthesia at these concentrations was approximately 164 s. In relation to the recovery time, all concentrations of EOs and eugenol remained in the suggested range by PARK *et al.* (2009), the largest time being 355 s. The concentration of the EO of *A. triphylla* to anesthetize both albino and gray strains of *R. quelen* at 24ºC and pH 7.0 within 3 min was between 400-800 µL L^{-1} (PARODI *et al.*, 2014). The lowest concentration of the EO of *L. alba* that induced anesthesia in *R. quelen* within 3 min at 21ºC and pH 7.0 was 400 mg L^{-1} (around 500 µL L^{-1} because the density of this EO is of approximately 0.8) (CUNHA *et al.*, 2010a), and in *H. reidi* was 450 µL L^{-1} (CUNHA *et al.*, 2011). Therefore, for both EOs deep anesthesia can be obtained with lower concentration in *S. rhombeus* than in these other species studied, probably due to the higher temperature used in the present study as found by GOMES *et al.* (2011). In contrast, *S. rhombeus* exposed to 50 µL L^{-1} did not reach anesthesia stage, but *H. reidi* did (CUNHA *et al.*, 2011). TONI *et al.* (2015) observed that the lowest concentration required to induce deep anesthesia in *Sparus aurata* at 38 ppt salinity, 18ºC, within 3 min was 200 µL L^{-1} EO of *L. alba*, the same concentration found to *S. rhombeus*. As for *S. rhombeus*, all concentrations tested in *S. aurata* showed recovery time lower than 10 min (TONI *et al.*, 2015).

The lowest eugenol concentration necessary to anesthetize *S. rhombeus* within 3 min was 50 µL L^{-1}. This is the same lowest eugenol concentration to anesthetize *R. quelen* (at 21°C and pH 7) (CUNHA *et al.*, 2010b), *B. amazonicus* (*B. cephalus*) (VIDAL *et al.*, 2007) and *P. mesopotamicus* (at 25 °C) (GONÇALVES *et al.*, 2008). *Centropomus parallelus* needed a similar eugenol concentration range to anesthetize and recover within the proposed periods: 25 – 62.5 mg L^{-1} (23.6 – 59 µL L^{-1}, because the density of eugenol is approximately 1.06) at 21°C (SOUZA *et al.*, 2012). *Brycon hilarii* needed higher concentrations than *S. rhombeus*, in the range of 100 – 300 mg L^{-1} (94.3 – 283 µL L^{-1}), for induction and recovery at the suitable time at 25°C (FABIANI *et al.*, 2013). In contrast, BITTENCOURT *et al.* (2012) verified that 75 mg L^{-1} (70.7 µL L^{-1}) eugenol required more than 3 min to anesthetize *C. auratus* (higher concentrations were not tested). The 5.0-7.0 pH range at 23ºC did not change time of induction to eugenol in *R. quelen* exposed to 40 mg L^{-1} (37.7 µL L^{-1}), but at pH 7.0 and 30ºC eugenol anesthetized this species within 225-275 s (depending on size of the fish) (GOMES *et al.*, 2011). *S. rhombeus* needs a higher time (443 s) to anesthetize at this eugenol concentration.

In the second experiment, the exposure of the fish to low concentrations of the anesthetics was performed to verify the possibility of using these products in the transport of *S. rhombeus*. The sedation stage, with lower responsiveness to external stimuli and metabolic rate, but without losing equilibrium, is recommended for transporting fish (SUMMERFELT and SMITH, 1990; PIRHONEN and SCHRECK, 2003).

Ethanol initially reduced swimming activity, but it returned to control level after 10 min. Ethanol enhances the action of several GABA receptors subtypes (WALLNER *et al.*, 2003), which may be related to the decreased swimming activity in *S. rhombeus*. This effect may have been fast due to ethanol evaporation. Both concentrations of EO of *A. triphylla* can be tested for transport of *S. rhombeus*, and the best concentration apparently is 10 µL L^{-1}, which reduced swimming activity through the 15 min observation. The addition of 30 to 50 µL L^{-1} of EO of *A. triphylla* in transport water (lower concentrations were not tested) reduced ions loss, plasma cortisol levels and ammonia excretion in *R. quelen*, suggesting lower physiological damage resulting from transport (PARODI *et al.*, 2014; ZEPPENFELD *et al.*, 2014). Both EO of *L. alba* concentrations can be tested for the transport of *S. rhombeus*. Similar concentrations (10 and 20 µL L^{-1}) of *L. alba* EO are recommended for the transport of *R. quelen* because they improved blood and ionoregulatory parameters (BECKER *et al.*, 2012). Both concentrations of eugenol can be tested for the transport of *S. rhombeus*, and the best concentration seems to be 5 µL L^{-1}, which reduced swimming activity at all observation times. The addition of 1 – 3 µL L^{-1} of eugenol is recommended for the transport of *R. quelen* because they decreased non-ionized ammonia levels, ion loss and mortality (BECKER *et al.*, 2012, 2013). The determination of the swimming activity allowed a more precise analysis of the sedative effects of the anesthetics tested, because the identification of the decreased reactivity to external stimuli proposed by SMALL (2003) for stage I is rather subjective. However, since this study analyzed swimming activity for only 15 min, it would be interesting that further studies investigate if the effects on swimming activity and equilibrium caused by the essential oils and eugenol can last for several hours, as well as if they can improve water, blood and ionoregulatory parameters as observed in the transport of silver catfish as observed by BECKER *et al.* (2012, 2013), PARODI *et al.* (2014) and ZEPPENFELD *et al.* (2014).

CONCLUSION

In conclusion, both *A. triphylla* and *L. alba* EOs and eugenol are effective for anesthetic induction within 3 min at the concentrations 150, 200 e 50 µL L^{-1}, respectively. For fast sedation, the recommended concentration is 50 µL L^{-1} for both EOs and 25 µL L^{-1} for eugenol. The concentrations of 5 and 10 µL L^{-1} of both EOs and eugenol reduced fish swimming activity and are indicated for studies of transportation of *S. rhombeus* but for eugenol and the EO of *A. triphylla* the best concentrations are 5 and 10 µL L^{-1}, respectively.

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REFERENCES

ANDERSON, W.G.; MCKINLEY, R.S.; COLAVECCHIA, M. 1997 The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. *North American Journal of Fisheries Management*, 17(2): 301-307. **http://dx.doi.org/10.1577/1548-8675(1997)017<0301:TUO COA>2.3.CO;2.**

ANJOS, H.D.B.; AMORIM, R.M.S.; SIQUEIRA, J.A.; ANJOS, C.R. 2009 Exportação de peixes ornamentais do estado do Amazonas, bacia Amazônica, Brasil. *Boletim do Instituto de Pesca*, 35(2): 259-274.

BECKER, A.G.; CUNHA, M.A.; GARCIA, L.O.; ZEPPENFELD, C.C.; PARODI, T.V.; MALDANER, G.; MOREL, A.F.; BALDISSEROTTO, B. 2013 Efficacy of eugenol and the methanolic extract of *Condalia buxifolia* during the transport of the silver catfish *Rhamdia quelen*. *Neotropical Ichthyology*, 11(3): 675-681. **http://dx.doi.org/10.1590/ S1679-62252013000300021.**

BECKER, A.G.; PARODI, T.V.; HELDWEIN, C.G.; ZEPPENFELD, C.C.; HEINZMANN, B.M.; BALDISSEROTTO, B. 2012 Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*. *Fish Physiology and Biochemistry*, 38(3): 789-796. PMid:21972065. **http://dx.doi.org/10.1007/s10695-011-9562-4.**

BENOVIT, S.C.; SILVA, L.L.; SALBEGO, J.; LORO, V.L.; MALLMANN, C.A.; BALDISSEROTTO, B.; FLORES, E.M.M.; HEINZMANN, B.M. 2015 Anesthetic activity and bio-guided fractionation of the essential oil of *Aloysia gratissima* (Gillies & Hook.) Tronc. in silver catfish *Rhamdia quelen*. *Anais da Academia Brasileira de Ciencias*, 87(3): 1675-1689. PMid:26221984. **http://dx.doi.org/10.1590/0001-3765201520140223.**

BITTENCOURT, F.; SOUZA, B.E.; BOSCOLO, W.R.; RORATO, R.R.; FEIDEN, A.; NEU, D.H. 2012 Benzocaína e eugenol como anestésicos para quinguio (*Carassius auratus*). *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 64(6): 1597-1602. **http://dx.doi.org/10.1590/ S0102-09352012000600028.**

CÁRDENAS, C.; TONI, C.; MARTOS-SITCHA, J.A.; CÁRDENAS, S.; HERAS, V.; BALDISSEROTTO, B.; HEINZMANN, B.M.; VÁZQUEZ, R.; MANCERA, J.M. 2016 Effects of clove oil, essential oil of *Lippia alba* and 2-phe anaesthesia on juvenile meagre, *Argyrosomus regius* (Asso, 1801). *Journal of Applied Ichthyology*, 32(4): 693-700. **http:// dx.doi.org/10.1111/jai.13048.**

CUNHA, M.A.; ZEPPENFELD, C.C.; GARCIA, L.O.; LORO, V.L.; FONSECA, M.B.; EMANUELLI, T.; VEECK, A.P.L.; COPATTI, C.E.; BALDISSEROTTO, B. 2010a Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. *Ciência Rural*, 40(10): 2107-2114. **http://dx.doi.org/10.1590/ S0103-84782010001000009.**

CUNHA, M.A.; BARROS, F.M.C.; GARCIA, L.O.; VEECK, A.P.L.; HEINZMANN, B.M.; LORO, V.L.; EMANUELLI, T.; BALDISSEROTTO, B. 2010b Essential oil of *Lippia alba*: a new anesthetic for silver catfish, *Rhamdia quelen. Aquaculture*, 306(1-4): 403-406. **http:// dx.doi.org/10.1016/j.aquaculture.2010.06.014.**

CUNHA, M.A.; SILVA, B.F.; DELUNARDO, F.A.C.; BENOVIT, S.C.; GOMES, L.C.; HEINZMANN, B.M.; BALDISSEROTTO, B. 2011 Anesthetic induction and recovery of *Hippocampus reidi* exposed to the essential oil of *Lippia alba. Neotropical Ichthyology*, 9(3): 683-688. **http://dx.doi.org/10.1590/S1679-62252011000300022.**

EUROPEAN PHARMACOPOEIA. 2007 European directorate for the quality of medicines. 6th ed. Strassbourg: European Pharmacopoeia.

FABIANI, B.M.; BOSCOLO, W.R.; FEIDEN, A.; DIEMER, O.; BITTENCOURT, F.; NEU, D.H. 2013 Benzocaine and eugenol as anesthetics for *Brycon hilarii. Acta Scientiarum Animal Science*, 35(2): 113-117.

FIGUEIREDO, R.O.; STEFANINI, M.B.; MING, L.C.; MARQUES, M.O.M.; FACANALI, R. 2004 Essential oil composition of *Aloysia triphylla* (L'Herit) Britton leaves cultivated in Botucatu, São Paulo, Brazil. *Acta Horticulturae*, 629(1): 131-134. **http://dx.doi.org/10.17660/ ActaHortic.2004.629.17.**

FREIRE, C.M.M.; MARQUES, M.O.M.; COSTA, M. 2006 Effects of seasonal variation on the central nervous system activity of *Ocimum gratissimum* L. essential oil. *Journal of Ethnopharmacology*, 105(1-2): 161-166. PMid:16303272. **http://dx.doi.org/10.1016/j.jep.2005.10.013.**

GOMES, D.P.; CHAVES, B.W.; BECKER, A.G.; BALDISSEROTTO, B. 2011 Water parameters affect anaesthesia induced by eugenol in silver catfish, *Rhamdia quelen. Aquaculture Research*, 42(6): 878-886. **http:// dx.doi.org/10.1111/j.1365-2109.2011.02864.x.**

GOMES, P.B.; FEITOSA, M.L.; SILVA, M.I.; NORONHA, E.C.; MOURA, B.A.; VENÂNCIO, E.T.; RIOS, E.R.; SOUSA, D.P.; VASCONCELOS, S.M.; FONTELES, M.M.; SOUSA, F.C. 2010 Anxiolytic-like effect of the monoterpene 1,4-cineole in mice. *Pharmacology, Biochemistry, and Behavior*, 96(3): 287-293. PMid:20670917. **http://dx.doi. org/10.1016/j.pbb.2010.05.019.**

GONÇALVES, A.F.N.; SANTOS, E.C.C.; FERNANDES, J.B.K.; TAKAHASHI, L.S. 2008 Mentol e eugenol como substitutos da benzocaína na indução anestésica de juvenis de pacu. *Acta Scientiarum. Animal Science (Penicuik, Scotland)*, 30(3): 339-344.

GUZMÁN-GUTIÉRREZ, S.L.; GÓMEZ-CANSINO, R.; GARCÍA-ZEBADÚA, J.C.; JIMÉNEZ-PÉREZ, N.C.; REYES-CHILPA, R. 2012 Antidepressant activity of *Litsea glaucescens* essential oil: Identification of ß-pinene and linalool as active principles. *Journal of Ethnopharmacology*, 143(2): 673-679. PMid:22867633. **http:// dx.doi.org/10.1016/j.jep.2012.07.026.**

HELDWEIN, C.G.; SILVA, L.L.; GAI, E.Z.; ROMAN, C.; PARODI, T.V.; BÜRGER, M.E.; BALDISSEROTTO, B.; FLORES, E.M.M.; HEINZMANN, B.M. 2014 *S*-(+)-Linalool from *Lippia alba*: sedative and anesthetic for silver catfish (*Rhamdia quelen*). *Veterinary Anaesthesia and Analgesia*, 41(6): 621-629. PMid:24628858. **http:// dx.doi.org/10.1111/vaa.12146.**

HELDWEIN, C.G.; SILVA, L.L.; RECKZIEGEL, P.; BARROS, F.M.C.; BÜRGER, M.E.; BALDISSEROTTO, B.; MALLMANN, C.A.; SCHMIDT, D.; CARON, B.O.; HEINZMANN, B.M. 2012 Participation of the GABAergic system in the anesthetic effect of *Lippia alba* (Mill.) N.E. Brown essential oil. *Brazilian Journal of Medical and Biological Research*, 45(5): 436-443. PMid:22473320. **http://dx.doi. org/10.1590/S0100-879X2012007500052.**

HOHLENWERGER, J.C.; COPATTI, C.E.; SENA, A.C.; COUTO, R.D.; BALDISSEROTTO, B.; HEINZMANN, B.M.; CARON, B.O.; SCHMIDT, D. 2016 Could the essential oil of *Lippia alba* provide a readily available and cost-effective anaesthetic for Nile tilapia (*Oreochromis niloticus*)? *Marine and Freshwater Behaviour and Physiology*, *49*(2): 119-126. **http://dx.doi.org/10.1080/10236244.2015.1123869.**

JÉGU, M.; INGENITO, L.F.S. 2007 Serrasalminae. In: BUCKUP, P.A.; MENEZES, N.A.; GHAZZI, M.S. *Catálogo das espécies de peixes de água doce do Brasil*. Rio de Janeiro: Museu Nacional. p. 40-43.

MOL, J.H. 2006 Attacks on humans by the piranha *Serrasalmus rhombeus* in Suriname. *Studies on Neotropical Fauna and Environment*, *41*(3): 189-195. **http://dx.doi.org/10.1080/01650520600630683.**

MPA – Ministério da Pesca e Aquicultura. 2011 *Boletim estatístico da pesca e aquicultura*. Brasília: MPA. 59 p.

NIST; EPA; NIH. 2010 *Mass spectral library and search/ analysis programs*. Hoboken: J. Willey and Sons.

PARK, I.S.; PARK, M.O.; HUR, J.M.; KIM, D.S.; CHANG, Y.J.; KIM, Y.J.; PARK, J.Y.; JOHNSON, S.C. 2009 Anesthetic effects of lidocaine hydrochloride on water parameters in simulated transport experiment of juvenile winter flounder *Pleuronectes americanus*. *Aquaculture*, *294*(1-2): 76-79. **http://dx.doi.org/10.1016/j.aquaculture.2009.05.011.**

PARODI, T.V.; CUNHA, M.A.; BECKER, A.G.; ZEPPENFELD, C.C.; MARTINS, D.I.; KOAKOSKI, G.; BARCELLOS, L.G.; HEINZMANN, B.M.; BALDISSEROTTO, B. 2014 Anesthetic activity of the essential oil of *Aloysia triphylla* and effectiveness in reducing stress during transport of albino and gray strains of silver catfish, *Rhamdia quelen*. *Fish Physiology and Biochemistry*, *40*(2): 323-334. PMid:23974669. **http://dx.doi.org/10.1007/s10695-013-9845-z.**

PARODI, T.V.; SANTOS, C.A.; VERONEZ, A.; GOMES, L.C.; HEINZMANN, B.M.; BALDISSEROTTO, B. 2016 Anesthetic induction and recovery time of *Centropomus parallelus* exposed to the essential oil of *Aloysia triphylla*. *Ciência Rural*, *46*(12): 2142-2147. **http://dx.doi.org/10.1590/0103-8478cr20160039.**

PAULUS, D.; VALMORBIDA, R.; TOFFOLI, E.; NAVA, G.A.; PAULUS, E. 2013 Teor e composição química do óleo essencial e crescimento vegetativo de *Aloysia triphylla* em diferentes espaçamentos e épocas de colheita. *Revista Ceres*, *60*(3): 372-379. **http://dx.doi.org/10.1590/S0034-737X2013000300010.**

PIRHONEN, J.; SCHRECK, C.B. 2003 Effects of anesthesia with MS-222, clove oil and CO2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). *Aquaculture (Amsterdam, Netherlands)*, *220*(1-4): 507-514. **http://dx.doi.org/10.1016/S0044-8486(02)00624-5.**

SARTORATTO, A.; MACHADO, A.L.M.; DELARMELINA, C.; FIGUEIRA, G.M.; DUARTE, M.C.T.; REHDER, V.L.G. 2004 Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Brazilian Journal of Microbiology*, *35*(4): 275-280. **http://dx.doi.org/10.1590/S1517-83822004000300001.**

SENA, A.C.; TEIXEIRA, R.R.; FERREIRA, E.L.; HEINZMANN, B.M.; BALDISSEROTTO, B.; CARON, B.O.; SCHMIDT, D.; COUTO, R.D.; COPATTI, C.E. 2016 Essential oil from *Lippia alba* has anaesthetic activity and is effective in reducing handling and transport stress in tambacu (*Piaractus mesopotamicus×Colossoma macropomum*). *Aquaculture (Amsterdam, Netherlands)*, *465*(1): 374-379. **http://dx.doi.org/10.1016/j.aquaculture.2016.09.033.**

SILVA, E.M.P.; OLIVEIRA, R.H.F.; RIBEIRO, M.A.R.; COPPOLA, M.P. 2009 Efeito anestésico do óleo de cravo em alevinos de lambari. *Ciência Rural*, *39*(6): 1851-1856. **http://dx.doi.org/10.1590/S0103-84782009005000127.**

SMALL, B.C. 2003 Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*. *Aquaculture (Amsterdam, Netherlands)*, *218*(1-4): 177-185. **http://dx.doi.org/10.1016/S0044-8486(02)00302-2.**

SOUZA, R.A.R.; CARVALHO, C.V.A.; NUNES, F.F.; SCOPEL, B.R.; GUARIZI, J.D.; TSUZUKI, M.Y. 2012 Efeito comparativo da benzocaína, mentol e eugenol como anestésicos para juvenis de robalo peva. *Boletim do Instituto de Pesca*, *38*(3): 247-255.

SUMMERFELT, R.C.; SMITH, L.S. 1990 Anaesthesia, surgery, and related techniques. In: SCHRECK, C.B.; MOYLE, P.B. *Methods for fish biology*. Bethesda: American Fisheries Society. p. 213-272.

TEIXEIRA, R.R.; SOUZA, R.C.; SENA, A.C.; BALDISSEROTTO, B.; HEINZMANN, B.M.; COUTO, R.D.; COPATTI, C.E. 2017 Essential oil of *Aloysia triphylla* in Nile tilapia: anaesthesia, stress parameters and sensory evaluation of fillets. *Aquaculture Research*, *48*(7): 3383-3392. **http://dx.doi.org/10.1111/are.13165.**

TONI, C.; BECKER, A.G.; SIMÕES, L.N.; PINHEIRO, C.G.; SILVA, L.L.; HEINZMANN, B.M.; CARON, B.O.; BALDISSEROTTO, B. 2014 Fish anesthesia: effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). *Fish Physiology and Biochemistry*, *40*(3): 701-714. PMid:24141557. **http://dx.doi.org/ 10.1007/s10695-013-9877-4.**

TONI, C.; MARTOS-SITCHA, J.A.; BALDISSEROTTO, B.; HEINZMANN, B.M.; SILVA, L.L.; MARTÍNEZ-RODRÍGUEZ, G.; MANCERA, J.M. 2015 Sedative effect of 2-phenoxyethanol and essential oil of *Lippia alba* on stress response in gilthead sea bream (*Sparus aurata*). *Research in Veterinary Science*, *103*(1): 20-27. PMid:26679791. **http://dx.doi.org/10.1016/j.rvsc.2015.09.006.**

VALE, T.G.; FURTADO, E.C.; SANTOS JUNIOR, J.G.; VIANA, G.S. 2002 Central effects of citral, myrcene and limonene, constituents of essential oil chemotypes from *Lippia alba* (Mill.) N.E. Brown. *Phytomedicine*, *9*(8): 709-714. PMid:12587690. **http://dx.doi.org/10.1078/094471102321621304.**

VIDAL, L.V.O.; FURUYA, W.M.; GRACIANO, T.S.; SCHAMBER, C.R.; SILVA, L.C.R.; SANTOS, L.D.; SOUZA, S.R. 2007 Eugenol como anestésico para juvenis de matrinxã (*Brycon cephalus*). *Revista Brasileira de Saúde e Produção Animal*, *8*(4): 335-342.

WALLNER, M.; HANCHAR, H.J.; OLSEN, R.W. 2003 Ethanol enhances 4 3 and 6 3 -aminobutyric acid type A receptors at low concentrations known to affect humans. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(25): 15218-15223. PMid:14625373. **http://dx.doi.org/10.1073/pnas.2435171100.**

ZEPPENFELD, C.C.; TONI, C.; BECKER, A.G.; MIRON, D.S.; PARODI, T.V.; HEINZMANN, B.M.; BARCELLOS, L.J.G.; KOAKOSKI, G.; ROSA, J.G.S.; LORO, V.L.; CUNHA, M.A.; BALDISSEROTTO, B. 2014 Physiological and biochemical responses of silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton. *Aquaculture*, *418-419*(1): 101-107. **http://dx.doi.org/10.1016/j.aquaculture.2013.10.013.**

3.2 MANUSCRITO 2

Manuscrito a ser resubmetido a Neotropical Ichthyology

Essentials oils of *Aloysia triphylla* and *Lippia alba* as stress-reducing and anesthetics for *Serrasalmus eigenmanni*

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Abstract

This study evaluated if the acidic pH and high temperature of the Amazonian blackwaters can alter the anesthetic and stress-reducing effects of the essential oils (EOs) of *Aloysia triphylla* and *Lippia alba*. The fish were placed in aquaria containing EOs of *A. triphylla* or *L. alba* (25 to 200 µL L^{-1}). Then, fish were transferred to aquaria containing EO-free water to evaluate the recovery time. In another experiment, fish were transferred to aquaria containing EOs of *A. triphylla* or *L. alba* (3 to 10 µL L^{-1}) and swimming behavior was analyzed up to 240 min of exposure. Water samples were collected at 0 and 240 min and blood samples at 240 min. The tested concentrations induced all stages of anesthesia, except 25 µL L^{-1} *A. triphylla* EO and 50 µL L^{-1} *L. alba* EO, which only induced sedation. The prolonged exposure to both EOs reduced swimming time compared to control in all evaluated times. The fish exposed to 3 µL L^{-1} *A. triphylla* EO showed lower net K$^+$ efflux compared to ethanol-exposed fish and in those

exposed to 5 µL L^{-1} ammonia excretion reduced. The blood parameters did not show significant difference among treatments.

Keywords: Amazonian blackwaters, Anesthesia, Ion fluxes, Piranha, Stress

Resumo

Este estudo avaliou se o pH ácido e a alta temperatura das águas negras amazônicas poderiam alterar os efeitos anestésico e redutor de estresse dos óleos essenciais (OEs) de *Aloysia triphylla* e *Lippia alba*. Os peixes foram colocados em aquários contendo OEs de *A. triphylla* ou *L. alba* (25 a 200 µL L^{-1}) para determinar o efeito anestésico. Após, peixes foram transferidos para aquários com água sem anestésicos para avaliar o tempo de recuperação. Em outro experimento, peixes foram transferidos para aquários contendo OEs de *A. triphylla* e *L. alba* (3 a 10 µL L^{-1}). Comportamento natatório foi analisado até 240 min de exposição. Foram coletadas amostras de água em 0 e 240 min e de sangue em 240 min. As concentrações testadas induziram todos os estágios de anestesia, exceto 25 µL L^{-1} OE de *A. triphylla* e 50 µL L^{-1} OE de *L. alba*, que causaram somente sedação. Exposição prolongada a ambos OEs reduziu o tempo de natação comparado ao controle. Peixes expostos a 3 µL L^{-1} OE de *A. triphylla* apresentaram menor efluxo de K^{+} comparado a expostos ao etanol e nos expostos a 5 µL L^{-1} a excreção de amônia reduziu. Parâmetros sanguíneos não diferiram entre tratamentos. CONCLUSÂO

Palavras-chave: Águas pretas da Amazônia, Anestesia, Estresse, Fluxo iônico, Piranha

Running head: Anesthetics for fish in Amazonian blackwaters

Introduction

Stress is a condition in which fish loses physiological homeostasis and can be lethal if not reversed. Exposure to stressors is unavoidable in aquaculture because fish are routinely submitted to practices such as handling, transportation, high stocking density, among others (Sampaio & Freire, 2016). Synthetic anesthetic substances have been used in order to prevent stress in fish (Pramod *et al*., 2010; Small, 2003; Weber *et al*., 2009). However, several of these substances can cause loss of mucus, increase of serum cortisol levels, immunosuppression, acidosis, tissue hypoxia (Zahl *et al*., 2012; Sneddon, 2012), and be aversive (Readman *et al*., 2013). Tricaine methanesulfonate (MS-222), the only anesthetic approved by the FDA for use in fish for consumption, induced hepatic lipoperoxidation in silver catfish *Rhamdia quelen* (Gressler *et al*., 2012). Due to adverse effects, there has been an increase in research involving natural products such as essential oils (EO) extracted from plants to replace the anesthetics of synthetic source. In addition, these products are allowed in organic aquaculture by Food and Agriculture Organization of the United Nations (FAO) (Bansemir et al., 2006) and also by Brazilian legislation (Brasil, 2011).

Studies have shown the efficacy in anesthesia and in prevention of stress damage of essential oils extracted from plants, such as: *Ocimum gratissimum* (Boijink *et al*., 2016; Silva *et al*., 2012), *Hesperozygis ringens* (Silva *et al*., 2013), *Nectandra megapotamica* (Tondolo *et al*., 2013), *Hyptis mutabilis* and *Ocotea acutifolia* (Silva *et al*., 2013). The EOs of *L. alba* and *A. triphylla* showed anesthetic efficacy and stress-reducing effect in *Centropomus parallelus* (Parodi *et al*., 2016), *R. quelen* (Cunha *et al*., 2010; Parodi *et al*., 2014; Becker *et al*., 2016), *Hyppocampus reidi* (Cunha *et al*., 2011) and did not induce aversiveness in fish (Bandeira Junior *et al.,* 2018). Almeida *et al*. (2018) demonstrated the anesthetic and sedative efficacy of *A. triphylla* and *L. alba* EOs in *Serrasalmus rhombeus*, an Amazonian fish living in the blackwaters of the Negro river, but did not evaluate if these EOs prevent stress damage such as ionoregulatory and blood disturbances when applied in blackwaters.

The Amazonian blackwaters have high concentration of humic and fulvic acids from the dissolved organic matter decomposition, resulting in acidic pH close to 5,0-6,0 (Küchler *et al*., 2000; Mortatti & Probst, 2003; Matsuo & Val, 2007) and with average annual temperature of 30,6°C (Fonseca *et al*., 1982). Knowing that the pH and temperature of the water can affect the anesthetic induction and recovery times (Gomes *et al*., 2011), the aim of this study was to evaluated if *A. triphylla* and *L. alba* EOs can be used as anesthetics and transport stress-reducing agents in fish that live in the Amazonian blackwaters.

Material and Methods

Essential oil extraction and analysis. The plants *A. triphylla* and *L. alba* were cultivated at the campus of the Universidade Federal de Santa Maria in the city of Frederico Westphalen, southern Brazil. A voucher specimen (SMDB No. 11169) was deposited in the herbarium of the Biology Department. The EO extraction and analysis was made as described previously by Almeida *et al*. (in press). The main compounds of the EO of *A. triphylla* were geranial (24.3%), limonene (21.7%) and cis-carveol (Z-carveol) (18.5%) and of the EO of *L. alba* were linalool (66.3%) and eucalyptol (10.6%) (Almeida *et al*., in press).

Animals. Specimens of *Serrasalmus eigenmanni* (171.01 ± 6.38 g; 18.26 ± 0.39 cm) (voucher number: INPA-ICT 53105) were collected during an expedition to Anavilhanas Islands of the Rio Negro, 110 km upstream from Manaus (2°23'41"S, 60°55'14"W). Fish were maintained in tanks supplied with flow trough water, pumped directly from Rio Negro (30°C, pH 5.1) continuously aerated for a few hours before testing.

Experiment I: Anesthesia induction and recovery in *Serrasalmus eigenmanni* exposed to *A. triphylla* and *L. alba* EOs. The fish were transferred individually to aquaria containing 5 L (30 ± 0.81°C, pH 5.1 ± 0.09) of water with the *L. alba* or *A. triphylla* EOs in the 25 – 200 μL L^{-1} concentration range first diluted in ethanol at a proportion of 1:10. These concentrations were chosen based on a previous study with these EOs in *S. rhombeus* (Almeida *et al*., 2018). The time for anesthesia induction was evaluated according to Small (2003): the stage I corresponds to sedation, when the reactivity to external stimuli decreased; in stage II occurs partial loss of equilibrium and erratic swimming and in stage III occurs total loss of equilibrium and cessation of locomotion. In recovery, the fish returns to regular swimming and reaction to external stimuli. Eight fish were used for each concentration tested and each fish was tested only once. The maximum observation time was 15 min. Control experiments was performed using aquaria containing only water and aquaria containing water and ethanol at a concentration equivalent to the highest dilution (1800 μL L^{-1}). After induction of anesthesia, fish were transferred to a tank containing water only to evaluate the recovery time. The animals were considered recovered when showed regular swimming and reaction to external stimuli (the peduncle caudal fin was pressed with a glass rod).

Experiment II: Prolonged exposure of *Serrasalmus eigenmanni* to the *A. triphylla* and *L. alba* EOs. The fish were placed into tanks containing 15 L of water and *A. triphylla* (3 and 5 µL L^{-1}) or *L. alba* (5 and 10 µL L^{-1}) EOs, first diluted in ethanol at a proportion of 1:10, and kept for 4 hours. Control experiments was performed using aquaria containing only water and aquaria containing water and ethanol at a concentration equivalent to the highest dilution (90 µL L^{-1}) These concentrations were chosen based on Almeida *et al*. (2018). Three fish per aquaria were used for each concentration (three replicates for each concentration).

Analyses of behavior

The fish were filmed for 30 s for analysis of the total swimming time and equilibrium (partial or total loss of equilibrium or normal) at 0, 5, 30, 60, 120, 180 and 240 min of exposure.

Water sampling and analyses

Water samples were collected at 0, 5, 30, 60, 120, 180 and 240 min of exposure. The total ammonia levels were checked according to Eaton *et al*. (2005). The levels of Na$^+$ and K$^+$ were determined with a flame photometer (Analyzer) and the chloride levels were determined using the colorimetric method described by Zall *et al*. (1956). The net ion fluxes and ammonia excretion were calculated according to Gonzalez *et al*. (1998): Jnet (ammonia excretion) = V ([ion]$_1$-[ion]$_2$) · ($M.t$)$^{-1}$, where [ion]$_1$ and [ion]$_2$ are the ion or ammonia concentrations in the water in which the fish were kept at the beginning and at the end of the experiment period, respectively; V is the volume of the water (in L); M is fish mass (in kg); and t the duration of the exposure (in h).

Blood sampling and analyses

After 240 min of exposure to EOs, blood samples were collected using heparinized syringes. The blood was centrifuged for five minutes at 665.1 g and the obtained plasma was frozen in liquid nitrogen. All these samples were kept in freezer at -80°C until further analysis. Plasma concentrations of cortisol (DBC *Diagnostics Biochem Canada*, ON, Canadá) and total thyroxine (T$_4$) (Symbiosys-Alka Tecnologia®, SP, BR) were quantified in triplicate by the enzyme immunoassay method (ELISA) in a microplate reader (Molecular Devices, CA, USA). Lactate was determined by reacting the lactate dehydrogenase enzyme (LDH Kit-Sigma Diagnostics, St. Louis, MO, USA) according to Gutmann and Wahlefeld (1974). Total lipids concentration was determined by colorimetric spectrophotometry method according to Frings *et al*. (1972), using cod liver oil (Sigma Diagnostics, St. Louis, MO, USA) as standard. The

reaction was measured at 540 nm. Total protein was determined by the colorimetric method described by Lowry *et al*. (1951), using bovine serum albumin (Sigma Diagnostics, St. Louis, MO, USA) as standard and the reaction was measured at 625 nm. Glucose was determined by enzymatic colorimetric method based on the oxidase/peroxidase reaction (Glucose LiquiColor Test-InVitro®, Fr).

Statistical analyses

All data were expressed as mean ± SEM. Homogeneity of variances among treatments was tested by Levene's test. Anesthetic induction and recovery times and net ion fluxes comparisons between treatments were assessed using the parametric o*ne-way* ANOVA and Tukey's test. When data violated the premises of ANOVA, the non-parametric Kruskal-Wallis was used for comparisons of mean ranks. The comparisons between the different treatments and swimming time were assessed using the non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test followed by the post-hoc Nemenyi test. The analysis was performed using the Statistica 7.0 software (Stat Soft. Inc.) and the minimum significance level was set at P<0.05.

Results

The concentration of 25 µL L^{-1} *A. triphylla* EO and 50 µL L^{-1} *L. alba* EO only induced sedative effect within 15 min, however all other EOs concentrations induced all stages of anesthesia. Fish exposed to 100 and 150 µL L^{-1} *A. triphylla* EO reached deep anesthesia (stage 3) significantly faster than the lower concentrations and the concentration of 100 µL L^{-1} *A. triphylla* EO showed lower recovery time (Fig. 1a). In the case of *L. alba* EO, fish reached the stage 3 with 100 and 200 µL L^{-1} and the recovery time did not differ at both concentrations (Fig. 1b). The exposure to 1800 µL L^{-1} ethanol alone did not produce any anesthetic effect.

In the second experiment, fish exposed to 3 and 5 µL L^{-1} EO of *A. triphylla* and 5 and 10 µL L^{-1} EO of *L. alba* presented lower swimming time than control fish over all analyzed times. Out of all fish exposed to 10 µL L^{-1} EO of *L. alba*, 22.22 % showed loss of equilibrium after 1 h exposure. The fish did not show loss of equilibrium in the other tested concentrations of both EOs. Ethanol-exposed fish showed lower swimming time compared to control in the first 5 min and in the last 60 min of exposure (Fig. 2). There was no significant difference in net ion (Na$^+$, K$^+$ and Cl$^-$) fluxes, except at 3 µL L^{-1} *A. triphylla* EO, in which the K$^+$ efflux was lower compared to ethanol-exposed fish (Fig. 3). Ammonia excretion did not differ between treatments, except at the 5 µL L^{-1} *A. triphylla* EO, in which ammonia excretion was not detected (Fig. 4). The plasma levels of cortisol, total T4, glucose, lactate and lipid showed no significant

differences over all treatments. Protein levels decreased in fish exposed to 5 µL L^{-1} *L. alba* EO compared to control and ethanol (Tab. 1).

Discussion

Fish are exposed to several stressors during their life cycle both in the natural environment and in fish farms. Anesthetic substances are used to prevent or reduce stress damage in cultured fish. According to Ross E Ross (2008), the maximum acceptable time to induce deep anesthesia is 600 sec. The present study showed that both EOs induced deep anesthesia in *S. eigenmanni* in less than 600 sec, therefore they can be used as anesthetic in this species. The lower concentrations of *A. triphylla* and *L. alba* EOs, 25 µL L^{-1} and 50 µL L^{-1} respectively, induced only sedation stage, in which the fish present low reactivity to external stimuli and partial loss of equilibrium.

The best concentration for deep anesthesia in *S. eigenmanni* is 100 µL L^{-1} *A. triphylla* EO with induction time of 266 s and recovery time of 110 s. The recommended concentration for deep anesthesia in both albino and gray strains of *R. quelen* and in *C. parallelus* is 200 µL L^{-1} with the same EO (Parodi *et al*. 2014; 2016). These species need a concentration higher than that required for deep anesthesia of *S. eigenmanni*. In the case of *L. alba* EO, the best concentration to induce deep anesthesia in *S. eigenmanni* is also 100 µL L^{-1}, with induction and recovery times of 236 s and 242 s, respectively. *Serrasalmus eigenmanni* needs lower *L. alba* EO concentration for the same anesthesia induction time compared to Nile tilapia *Oreochromis niloticus* (around 450 µL L^{-1}) (Hohlenwerger *et al*., 2016) and silver catfish *R. quelen* (around 450 µL L^{-1}) (Cunha *et al*., 2010), but similar to tambacu, the hybrid of *Piaractus mesopotamicus* × *Colossoma macropomum* (Sena *et al.*, 2016).

In the second experiment, low concentrations were tested to verify the viability of the use these EOs in the transport of *S. eigenmanni*. The lower swimming activity of the fish exposed to the EOs is an indicative that these products can be recommended for the transport of Amazon fishes at high temperatures. For the fish transport, it is recommended that the animals remain in deep sedation stage, in which there is loss of responsiveness to external stimuli but without loss of equilibrium and supposedly with lower metabolic activity (Summerfelt E Smith, 1990; Pirhonen E Schreck, 2003). The equilibrium loss of 22.22 % of fish after 1 h of exposure suggests that the concentration of 10 µL L^{-1} of *L. alba* EO cannot be used in fish transport at high temperatures, because according to Cooke *et al*. (2014) in this stage the fish may die by suffocation.

In stressful situations, the organism responds with sympathetic activation of the hypothalamic-hypophysis-interrenal (HHI) axis that triggers a change in the blood hormone levels, mainly catecholamines secreted by the chromaffin cells and the release of cortisol by the interrenal tissue (Mazeaud *et al.*, 1977; Barton and Iwama, 1991; Barton 2002). Consequently, metabolic changes as the increase of glucose, lactate, and thyrosine (T4) and decrease in protein levels as well as ionoregulatory disorders (Barton, 2002) may occur.

The addition of EO of *A. triphylla* and EO of *L. alba* in transport water of silver catfish *R. quelen* showed that these EOs minimized stress transport effects through reduction of cortisol release, ammonia excretion and ions loss (Becker *et al.* 2012, 2013; Parodi *et al.*, 2014; Zeppenfeld *et al.* 2014). The addition of 20 µL L^{-1} of *A. triphylla* EO in transport water of *C. paralellus* decreased the cortisol plasma levels and ammonia excretion (Parodi *et al.*, 2016). However, in the present study the metabolic parameters, ammonia excretion, and ions fluxes were not affected by exposure to EOs, except at 3 and 5 µL L^{-1} of *A. triphylla* EO, in which there was a reduction of K$^+$ efflux and ammonia excretion, respectively. The decrease of ammonia excretion may be related to metabolism reduction, in accordance with the lower swimming activity in *S. eigenmanni* provided by this EO. Lower ammonia excretion was also observed in silver catfish (Zeppenfeld *et al.*, 2014) and *C. paralellus* transported with this EO (Parodi *et al.*, 2016). On the other hand, Mazandarani *et al.* (2016) attributed the low ammonia excretion associated with high serum urea level in common carp *Cyprinus carpio* transported with linalool (the main constituent of the EO of *L. alba*) in the water as a sign of impaired gill function. Additional studies analyzing plasma ammonia and urea levels in fish transported with *A. triphylla* and *L. alba* EOs will shed light to this point.

Regarding the active substances, literature reports evidenced central depressor effects for geranial, limonene, and linalool in rodents *in vivo* and *ex vivo* models (Vale *et al.*, 1999; 2002; Sousa *et al.*, 2015) and also in fish for linalool (Heldwein *et al.*, 2014). In addition, carveol itself did not induce anesthesia, but enhanced propofol anesthetic effects in mice (Lin *et al.*, 2006). So, the available information indicates that the observed effects result from the interaction of different EO components.

In conclusion, both EOs showed anesthetic effects in *S. eigenmanni*. The concentrations recommended are 25 and 50 µL L^{-1} for sedation and 100 µL L^{-1} for deep anesthesia. The concentration 5 µL L^{-1} of both EOs is recommended for transport lasting up to 4 h of *S. eigenmanni*, because at this concentration fish decreased ammonia excretion with the EO of *A. triphylla* and maintained swimming equilibrium with both EOs.

References

Almeida APG, Heinzmann BM, Val AL, Baldisserotto, B. Essential oils and eugenol as anesthetics for *Serrasalmus rhombeus*. Bol Inst Pesca. 2018; 44(1):44-50.

Bandeira Junior G, Abreu MS, Rosa JGD, Pinheiro CG, Heinzmann BM, Caron BO, Baldisserotto B, Barcellos LJG. *Lippia alba* and *Aloysia triphylla* essential oils are anxiolytic without inducing aversiveness in fish. Aquaculture. 2018; 482:49-56.

Bansemir A, Blume M, Schröder S, Lindequist U. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture. 2006; 252: 79–84.

Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis. 1991; 10(1):3-26.

Barton BA. Stress in fish: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol. 2002; 42(3):517-525.

Becker AG, Parodi TV, Heldwein CG, Zeppenfeld CC, Heinzmann BM, Baldisserotto B. Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*. Fish Physiol Biochem 2012; 38(3):789-796.

Becker AG, Cunha MA, Garcia LO, Zeppenfeld CC, Parodi TV, Maldaner G, Morel AF, Baldisserotto B. Efficacy of eugenol and the methanolic extract of *Condalia buxifolia* during the transport of the silver catfish *Rhamdia quelen*. Neotrop Ichthyol 2013; 11(3):675-681.

Becker AG, Parodi TV, Zeppenfeld CC, Salbego J, Cunha MA, Heldwein CG, Loro VL, Heinzmann BM, Baldisserotto B. Pre-sedation and transport of *Rhamdia quelen* in water containing essential oil of *Lippia alba*: metabolic and physiological responses. Fish Physiol Biochem. 2016; 42(1):73-81.

Boijink CL, Queiroz CA, Chagas EC, Chaves FCM, Inoue LAKA. Anesthetic and anthelminthic effects of clove oil basil (*Ocimum gratissimum*) essential oil for tambaqui (*Colossoma macropomum*). Aquaculture. 2016; 457(1):24-28.

BRASIL. Instrução Normativa Interministerial nº. 28, de 8 de junho de 2011: estabelece normas técnicas para os sistemas orgânicos de produção aquícola a serem seguidos por toda pessoa

física ou jurídica responsável por unidades de produção em conversão ou por sistemas orgânicos de produção. Diário Oficial [da] República Federativa do Brasil, Brasília, DF, 09 jun. 2011. Seção 1, p. 04-09.

Cooke SJ, Suski CD, Ostrand KG, Tufts BL, Whal DH. Behavioral and physiological assessment of low concentrations of clove oil anaesthetic for handling and transporting largemouth bass (*Micropterus salmoides*). Aquaculture. 2004; 239(1-4):509-529.

Cunha MA, Barros FMC, Garcia LO, Veeck APL, Heinzmann BM, Loro VL, Emanuelli T, Baldisserotto B. Essential oil of *Lippia alba*: A new anesthetic for silver catfish, *Rhamdia quelen*. Aquaculture. 2010; 306(1-4):403-406.

Cunha MA, Silva BF, Delunardo FAC, Benovit SC, Gomes LC, Heinzmann BM, Baldisserotto B. Anesthetic induction and recovery of *Hippocampus reidi* exposed to the essential oil of *Lippia alba*. Neotrop Ichthyol. 2011; 9(3):683-688.

Fonseca OJM, Salem JI, Guarim VL. Poluição e autopurificação do Rio Negro nas cercanias de Manaus. Acta Amaz. 1982; 12(2):271-278.

Frings CS, Fendley TW, Dunn RT, Queen CA. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin Chem. 1972; 18(7):673-674.

Gomes DP, Chaves BW, Becker AG, Baldisserotto B. Water parameters affect anaesthesia induced by eugenol in silver catfish, *Rhamdia quelen*. Aquacult Res. 2011; 42(6):878-886.

Gonzalez RJ, Wood CM, Wilson RW, Patrick ML, Bergman HL, Narahara A, Val AL. Effects of water pH and calcium concentration on ion balance in fish of the Rio Negro, Amazon. Physiol Zool. 1998, 71(1):15-22.

Gressler LT, Riffel APK, Parodi TV, Saccol EMH, Koakoski G, Costa ST, Pavanato MA, Heinzmann BM, Caron B, Schmidt D, Llesuy SF, Barcellos LJG, Baldisserotto B. Silver catfish *Rhamdia quelen* immersion anaesthesia with essential oil of *Aloysia triphylla* (L'Hérit) Britton or tricaine methanesulfonate: effect on stress response and antioxidant status. Aquacult Res. 2012; 45(6):1061-1072.

Gutmann I, Wahlefeld W. L-(+)- Lactate. Determination with lactate dehydrogenase and NAD. In: H. U. Bergmeyer (Ed.), Methods of enzymatic analysis, New York: Verlag Chemie-Academic Press, 1974, p. 1464-1472.

Heldwein CG, Silva LL, Gai EG, Roman C, Parodi TV, Bürger, ME, Baldisserotto B, Flores EMM, Heinzmann BM. *S*-(+)-Linalool from *Lippia alba*: sedative and anesthetic for silver catfish (*Rhamdia quelen*). Vet Anaest Analg. 2014; 41 (6): 621-629.

Hohlenwerger JC, Copatti CE, Sena AC, Couto RD, Baldisserotto B, Heinzmann BM, Caron BO, Schmidt D. Could the essential oil of *Lippia alba* provide a readily available and cost-

effective anaesthetic for Nile tilapia (*Oreochromis niloticus*)? Mar Freshw Behav Physiol. 2016; 49(2):119-126.

Küchler IL, Miekeley N, Forsberg BR. A contribution to the chemical characterization of rivers in the Rio Negro basin, Brazil. J Braz Chem Soc. 2000; 11(3):286-292.

Lin AL, Shangari N, Chan TS, Remirez D, O'Brien PJ. Herbal monoterpene alcohols inhibit propofol metabolism and prolong anesthesia time. Life Sciences. 2006; 79, 21–29.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193(1):265-275.

Matsuo AYO, Val AL. Acclimation to humic substances prevents whole body sodium loss and stimulates branchial calcium uptake capacity in cardinal tetras *Paracheirodon axelrodi* (Schultz) subjected to extremely low pH. J Fish Biol. 2007; 70(1):989-1000.

Mazeaud MM, Mazeaud F, Donaldson EM. Primary and secondary effects of stress in fish: some new data with a general review. Trans Am Fish Soc. 1977; 106(3):201-212.

Mazandarani M, Hoseini SM, Ghomshani MD. Effects of linalool on physiological responses of *Cyprinus carpio* (Linnaeus, 1758) and water physico-chemical parameters during transportation. Aquacult Res. 2017; 48(12):5775-5781.

Mortatti J, Probst JL. Silicate rock weathering and atmospheric/soil CO_2 uptake in the Amazon basin estimated from river water geochemistry: seasonal and spatial variations. Chem Geol. 2003; 197(1-4):177-196.

Parodi TV, Cunha MA, Heldwein CG, Souza DM, Martins AC, Garcia LO, Junior WW, Monserrat JM, Schmidt D, Caron BO, Heinzmann BM, Baldisserotto B. The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloisya triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). Comp Biochem Physiol C Toxicol Pharmacol. 2012; 155(3):462-468.

Parodi TV, Cunha MA, Becker AG, Zeppenfeld CC, Martins DI, Koakoski G, Barcellos LG, Heinzmann BM, Baldisserotto B. Anesthetic activity of the essential oil of *Aloysia triphylla* and effectiveness in reducing stress during transport of albino and gray strains of silver catfish, *Rhamdia quelen*. Fish Physiol Biochem. 2014; 40(2):323-334.

Parodi TV, Santos CA, Veronez A, Gomes LC, Heinzmann BM, Baldisserotto B. Anesthetic induction and recovery time of *Centropomus parallelus* exposed to the essential oil of *Aloysia triphylla*. Cienc Rural. 2016; 46(12):2142-2147.

Pascual ME, Slowing K, Carretero E, Mata DS, Villar A. *Lippia*: traditional uses, chemistry and pharmacology: a review. J Ethnopharmacol. 2001; 76(3):201-214.

Pirhonen J, Schreck CB. Effects of anesthesia with MS-222, clove oil and CO_2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). Aquaculture. 2003; 220(1-4):507-514.

Pramod PK, Ramachandran A, Sajeevan TP, Thampy S, Pai SS. Comparative efficacy of MS-222 and benzocaine as anaesthetics under simulated transport conditions of a tropical ornamental fish *Puntius filamentosus* (Valenciennes). Aquacult Res. 2010; 41(2):309-314

Readman GD, Owen SF, Murrell JC, Knowles TG. Do fish perceive anaesthetics as aversive? PLos One. 2013; 8(9):e73773.

Ross GL, Ross BR. Anaesthetic and sedative techniques for aquatic animals. Oxford: Blacwell Publishing; 2008. Chapter 2, Defining stress in aquatic animals; p. 7-20.

Sampaio FDF, Freire CA. An overview of stress physiology of fish transport: changes in water quality as a function of transport duration. Fish Fish. 2016; 17(4):1055-1072.

Sena AC, Teixeira RR, Ferreira EL, Heinzmann BM, Baldisserotto B, Caron BO, Schmidt D, Couto RD, Copatti CE. Essential oil from *Lippia alba* has anaesthetic activity and is effective in reducing handling and transport stress in tambacu (*Piaractus mesopotamicus* x *Colossoma macropomum*). Aquaculture; 2016; 465:374-379.

Silva LL, Parodi TV, Reckziegel P, Garcia VG, Bürger ME, Baldisserotto B, Malmann CA, Pereira AMS, Heinzmann BM. Essential oil of *Ocimum gratissimum* L.: Anesthetic effects, mechanism of action and tolerance in silver catfish, *Rhamdia quelen*. Aquaculture. 2012; 350-353(1):91-97.

Silva LL, Silva DT, Garlet QI, Cunha MA, Mallmann CA, Baldisserotto B, Longhi SJ, Pereira AMS, Heinzmann BM. Anesthetic activity of Brazilian native plants in silver catfish (*Rhamdia quelen*). Neotrop Ichthyol. 2013; 11(2):443-451.

Sousa DG, Sousa SDG, Silva RER, Silva-Alves KS, Ferreira-da-Silva FW, Kerntopf MR, Menezes IRA, Leal-Cardoso JH, Barbosa R. Essential oil of *Lippia alba* and its main constituent citral block the excitability of rat sciatic nerves. Braz J Med Biol Res. 2015; 48 (8): 697-702.

Small BC. Anesthetic efficacy of metomitade and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*. Aquaculture. 2003; 218(1):177-185.

Sneddon LU. Clinical anesthesia and analgesia in fish. J Exot Pet Med. 2012; 21(1):32-43.

Summerfelt RC, Smith LS. Anaesthesia, surgery and related techniques. In: Schreck CB, Moyle PB, editors. Methods for fish biology. Bethesda, MD: American Fisheries Society; 1990. p. 213-272.

Tondolo JSM, Amaral LP, Simões LN, Garlet QI, Schindler B, Oliveira TM, Silva BF, Gomes LC, Baldisserotto B, Mallmann C, Heinzmann BM. Anesthesia and transport of fat snook *Centropomus parallelus* with the essential oil of *Nectandra megapotamica* (Spreng.) Mez. Neotrop Ichthyol. 2013; 11(3):667-674.

Weber RA, Peleteiro JB, Garcia Martin LO, Aldegunde M. The efficacy of 2-phenoxyethanol, metomidate, clove oil and MS-222 as anesthetic agents in the Senegalese sole (*Solea senegalensis* Kaup 1858). Aquaculture. 2009; 288(1-2):147-150.

Vale TG, Furtado EC, Santos Jr JG, Viana GS. Central effects of citral, myrcene and limonene, constituents of essential oil chemotypes from *Lippia alba* (Mill.) N.E. Brown. Phytomedicine. 2002; 9: 709-714.

Vale TG, Matos FJA, Lima TCM, Viana GSB. Behavioral effects of essential oils from *Lippia alba* (Mill.) N.E. Brown chemotypes. J Ethnopharmacol. 1999; 67(2): 127-133.

Zahl IH, Samuelsen O, Kiessling A. Anesthesia of farmed fish: Implications for welfare. Fish Physiol Biochem. 2012; 38(1):201-218.

Zall DM, Fisher, Garder QM. Photometric determination of chlorides in water. Anal Chem. 1956; 28(11):1665-1678.

Zeppenfeld CC, Toni C, Becker AG, Miron SD, Parodi TV, Heinzmann BM, Barcellos LJG, Koakosky G, Rosa JGS, Loro VL, Cunha MA, Baldisserotto B. Physiological and biochemical responses of silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton. Aquaculture. 2014; 418-419(1):101-107.
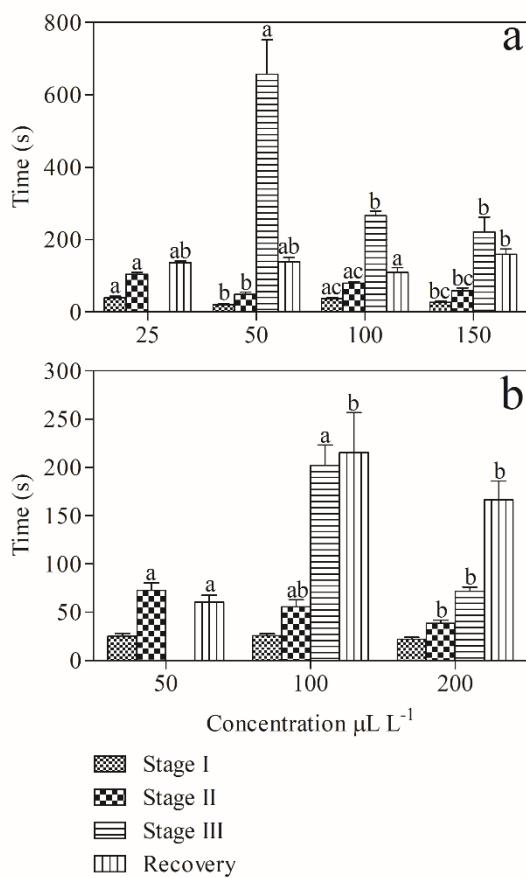
Fig. 1. Time required for induction and recovery of anesthesia in *Serrasalmus eigenmanni*. **a**: *Aloysia triphylla* essential oil. **b**: *Lippia alba* essential oil. Different letters indicate a significant difference between treatments based on one-way ANOVA and Tukey's test ($P < 0.05$).
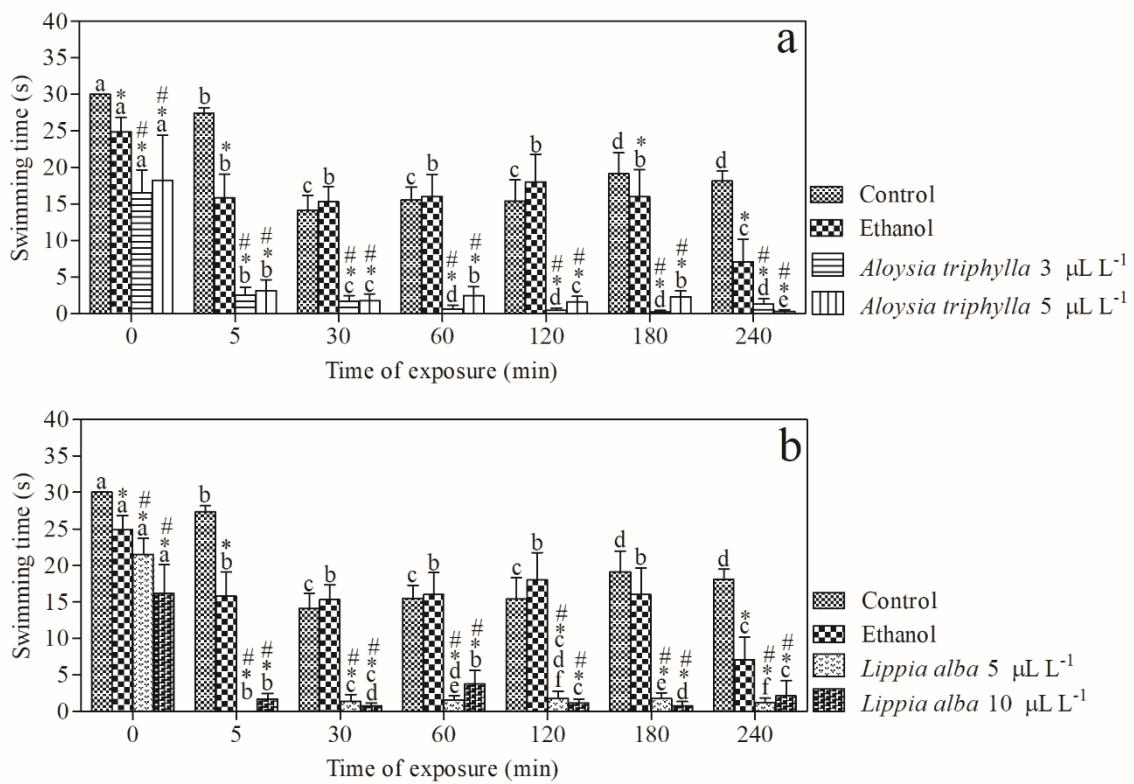
Fig. 2. Swimming time of *Serrasalmus eigenmanni* exposed to essential oils. **a:** *Aloysia triphylla*. **b:** *Lippia alba*. Different letters indicate significant difference between times of exposure based on non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test followed by the post-hoc Nemenyi test. * indicates significant difference from control fish. # indicates significant difference from ethanol-exposed fish.
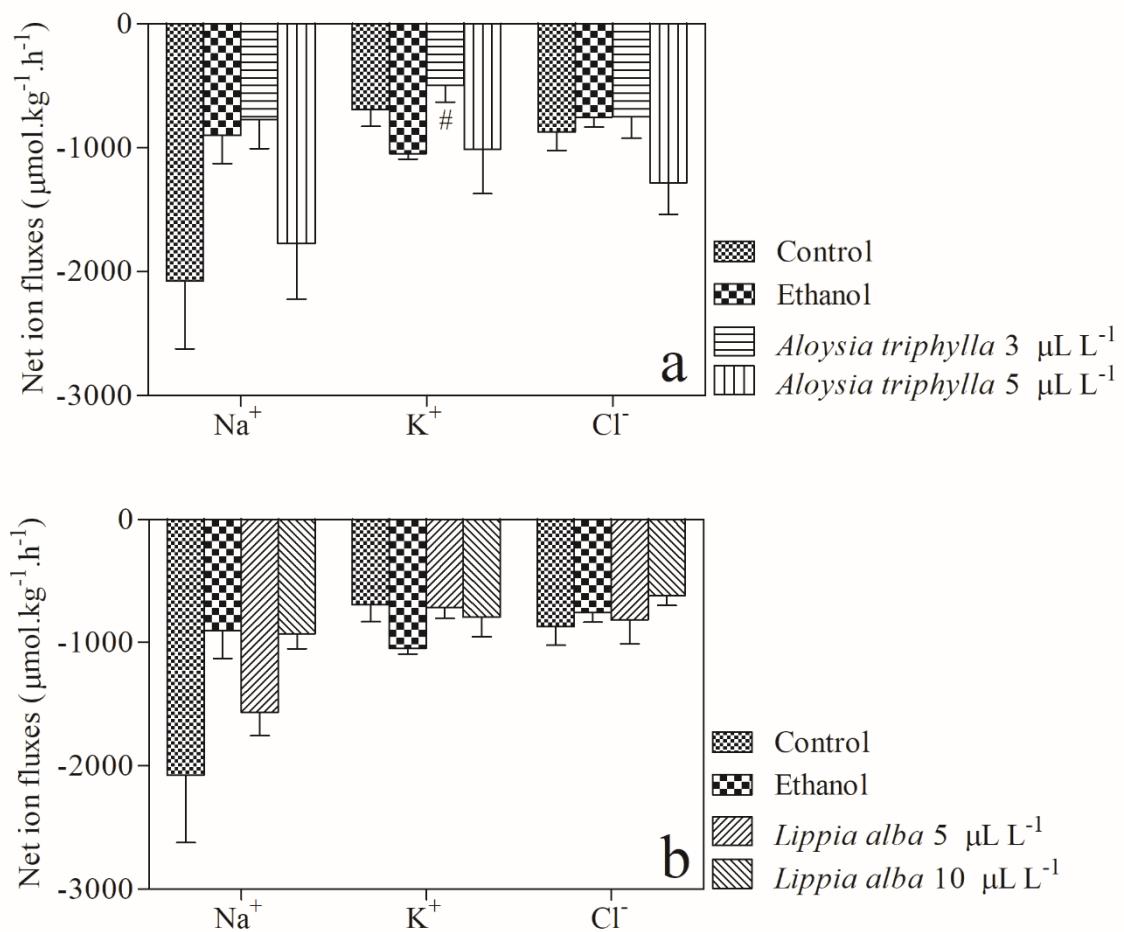
Fig. 3. Net ion fluxes of *Serrasalmus eigenmanni* exposed for 4 hours to essential oils. **a:** *Aloysia triphylla*. **b:** *Lippia alba*. # indicates significant difference from ethanol-exposed fish. Based on one-way ANOVA and Tukey's test (P < 0.05).
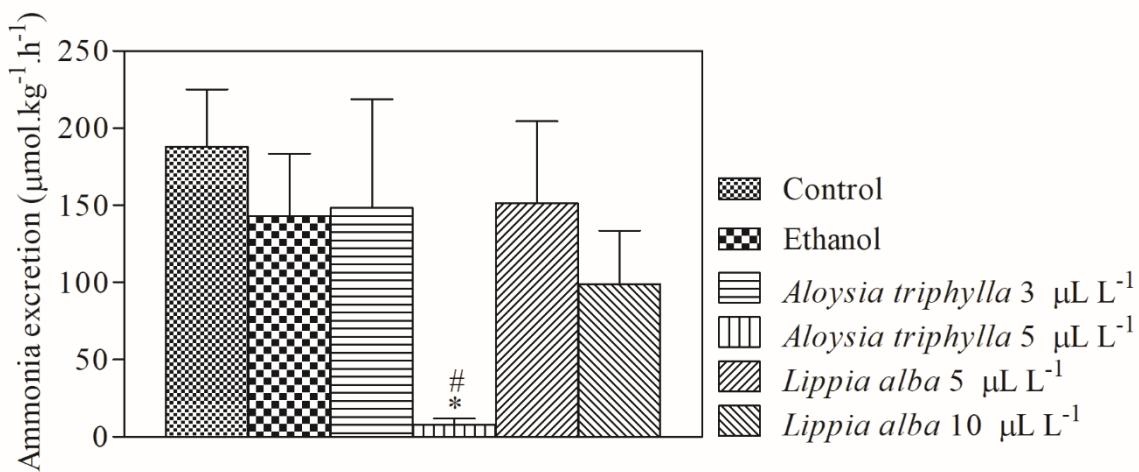
Fig. 4. Ammonia excretion of *Serrasalmus eigenmanni* exposed for 4 hours to essential oils. **a:** *Aloysia triphylla*. **b:** *Lippia alba*. * indicates significant difference from control fish. # indicates significant difference from ethanol-exposed fish. Based on one-way ANOVA and Tukey's test ($P < 0.05$).

Tab. 1. Plasma levels of cortisol (μg dL^{-1}), total T4 (μg dL^{-1}), glucose (mg dL^{-1}), lactate (mg dL^{-1}), protein (mg mL^{-1}) and lipids (mg mL^{-1}) in *Serrasalmus eigenmanni* exposed to different concentrations of essential oils of *Aloysia triphylla* and *Lippia alba*.

Treatment	Cortisol	Total T4	Glucose	Lactate	Protein	Lipid
Control	83.21 ± 16.06	15.55 ± 5.31	79.79 ± 8.98	21.10 ± 2.83	70.15 ± 2.40	11.65 ± 0.79
Ethanol	38.40 ± 5.07	17.64 ± 5.69	82.92 ± 7.02	63.34 ± 19.67	69.08 ± 6.57	11.28 ± 1.51
Lippia alba 5 μL L^{-1}	81.60 ± 6.48	12.55 ± 1.75	55.10 ± 4.92	35.78 ± 20.80	40.59 ± 4.46*#	11.03 ± 1.66
Lippia alba 10 μL L^{-1}	70.00 ± 9.90	20.90 ± 1.54	82.33± 5.76	31.07 ± 10.95	60.8 ± 10.19	11.80 ± 0.47
Aloysia triphylla 3 μL L^{-1}	47.22 ± 9.34	4.43 ± 2.70	64.32 ± 7.81	43.70 ± 22.62	67.57 ± 7.50	9.26 ± 0

* indicates significant difference from control fish. # indicates significant difference from ethanol-exposed fish. Based on one-way ANOVA and Tukey's test ($P < 0.05$).

3.3 MANUSCRITO 3

Daily rhythm of gene expression of digestive enzymes and clock genes in silver catfish *Rhamdia quelen*

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Abstract

The aim this study was evaluate daily rhythm of gene expression of digestive enzymes and clock genes in post-larvae and gastrointestinal tract of juveniles of *Rhamdia quelen* fed once daily and submitted to a 14L:10D photoperiod. The post-larvae were fed at 8 h, the first sampling was performed 1 h after feeding and the subsequent samplings, every 4 h. Post-larvae were stored in 1.5 ml RNase-free microcentrifuge tubes containing RNALater. Juveniles were fed at 8 h and samplings were performed 1, 3, 6, 12 and 24 h after feeding. The gastrointestinal tract was collected and stored in 15 ml RNase-free tubes containing RNALater. The gene expression of *pla2g1b*, *try2*, *pga2*, *ctr2 anrt1a*, *anrt1b*, *anrt1l*, *anrt2*, *clock, per1, per2, per3, cry1a, cry1b, cry2, cry3* and *amy2a* was measured by real-time PCR. The results showed that both post-larvae and juveniles did not present the typical interaction between the negative (*per* and *cry*) and the positive (*arntl* and *clock*) loops when sumitted to a LD photoperiod and only in post-larvae the gene expression of digestive enzymes is probably influenced by the expression of clock genes.

Keywords: *amy2a, arntl*, circadian cycle, *clock*, *ctr2, pga2, pla2g1b*, *try2*.

Introduction

All living organisms are influenced by various environmental cycles, which resulted in the development of an endogenous timing rhythm that coordinates biological functions with environmental conditions. The organisms have developed the ability to anticipate such

environmental changes, adapting their activities to the best time of day (MORGAN et al., 2004). The circadian rhythm is generated and regulated by positive and negative transcriptional-translational feedback loops of clock genes with a periodicity of approximately 24 hours. The positive loop is composed of the *circadian locomotor output cycles kaput* (*clock*) and *brain and muscle ARNT-like protein* (*bmal1*) genes which are transcription factors, whose proteins associate in the cytoplasm forming a heterodimer that translocate to the cellular nucleus and activates the negative loop genes. The negative loop is composed by *period* (*per1*, *per2* and *per3*) and *cryptochrome* (*cry1* and *cry2*) genes. In the cytoplasm, the proteins of these genes oligomerize and return to the nucleus to suppress the *clock*/*bmal1* complex (BELL-PEDERSEN et al., 2005; VATINE et al., 2011).

External factors capable of regulating endogenous cycle are known as zeitgebers. The most powerful and studied zeitgeber is the light/dark cycle (PANDA, HOGENESCH and KAY, 2002). However, when the food availability is restricted by time, feeding can act as an important zeitgeber. Studies with mammals and fish showed an increase in the locomotor activity of these animals hours before meals, behavior known as Food-Anticipatory Activity (FAA), when subjected to food restrictions (MISTLBERGER, 2009). However, it is not only behavioral activities that may be under the control of the circadian cycle, but also physiological and biochemical processes (PANDA, HOGENESCH and KAY, 2002). Studies evaluating the feeding schedule as synchronizer of daily rhythms demonstrated that fish fed daily at the same time showed changes in behavior, as well as in physiology such as variations in gastric lumen pH, blood glucose levels, enzymatic activity and the secretion of neuropeptide Y (VERA et al., 2007; MONTOYA et al, 2010). At the molecular level, studies demonstrated that feeding time may influence the gene expression of clock genes (FELICIANO et al., 2011; LÓPEZ-OLMEDA et al., 2010; MATA-SOTRES et al., 2015; SÁNCHEZ and SÁNCHEZ-VÁZQUEZ, 2009; VERA et al., 2013).

Silver catfish *Rhamdia quelen* is a freshwater siluriforme native from Central and South America (GOMES et al., 2000). It is an omnivorous fish (with tendency to carnivory) (KÜTTER, BENVENUTI and MORESCO, 2009) with diurnal habits (SCAGLIONE et al., 2018) and higher swimming activity in the morning and nightfall, and preference for shelters under wood debris (SCHULZ and LEUCHTENBERGER, 2006) or stones (GOMES et al., 2000). As a species of economic interest, studies on nutrition have been carried out in order to improve its performance. The utilization of the nutrients ingested depends on a digestive complex process, which has as key element the digestive enzymatic activity. There are studies evaluating the activity of digestive enzymes in *R. quelen* fed with different diet formulations

(GOULART et al., 2013; LAZZARI et al., 2010; MORO et al., 2010) as well as in specimens collected from the natural environment (ALMEIDA et al., 2018). However, there is no study evaluating at the molecular level these enzymes in this species, nor the influence of feeding time on the gene expression of digestive enzymes and clock genes. With the purpose of filling this gap, this study aimed to verify the daily rhythm of clock and digestive enzymes gene expression during a period of 24 h in post-larvae and juveniles of silver catfish *R. quelen*.

Material and Methods

Fish

Post-larvae (15 days after hatching) (0.068 ± 0.004 g) and juveniles (22.0 ± 1.087 g) of silver catfish *R. quelen* were purchased from a fish farm (Santa Maria/RS – Brazil) and transported to the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM). The post-larvae and juveniles were acclimated for two days in semi-static systems with 40-L and 250-L tanks, respectively, with biological filter, constant aeration and temperature of 22°C. The photoperiod in the acclimation period was 14h light: 10h dark (14L:10D). The fish were fed once daily with commercial feed at 8h and the tanks were siphoned once a day with 50% renewal of the water. There was no mortality during the acclimation period.

Experiment I

Post-larvae were distributed in six aquaria containing 10-L (n= 20 fish/aquarium) and kept under photoperiod 14L:10D. The post-larvae were fed at 08:00 h and samplings were performed every 4 h starting 1 h after the feeding (n= 10 fish/sampling) and euthanized by anesthetic overdose (100 µL L^{-1} *Cymbopogon flexuosus* EO) and then stored in 1.5 mL RNase-free microcentrifuge tubes containing 1.5 mL of RNA*Later*™ (Ambion®). During the nocturnal period, the samplings were performed with the minimum luminosity. The fish were sampled from the same aquaria in each sampling time to avoid stress.

Experiment II

Juveniles were distributed in five aquaria containing 30-L (n= 8 fish/aquarium). Fish were fed at 08:00 h and kept under photoperiod 14L:10D. Gastrointestinal tracts (GIT) (excluding liver) were sampled 1, 3, 6, 12, and 24 h after feeding. Fish were anesthetized (100 µL L^{-1} *Cymbopogon flexuosus* EO) and euthanized by spinal cord sectioning. The 8 fish were sampled from the same aquaria at each sampling to avoid stress. Samples were rinsed in 0.9 % saline

solution, weighed and stored in 15 mL tubes containing 10 volumes (w/v) of RNA*Later*™ (Ambion®). Surgical material and storage tubes were previously treated with UV light for decontamination.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the post-larvae and the gastrointestinal tracts of juveniles using NucleoSpin® RNA isolation kit (Macherey-Nagel), according to manufacturer's protocol. The concentration of total RNA was measured with a Qubit® 2.0 Fluorometer (Invitrogen™, Life Technologies) and its quality was assessed using an Agilent RNA 6000 Nano Assay Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNAs from post-larvae and gastrointestinal tracts (500 ng) samples were used to synthetize the first strand of cDNA by reverse transcription reaction using a qScript™ cDNA Synthesis Kit (Quanta BioSciences). The reverse-transcription was performed using a Mastercycler®proS vapo.protect thermal cycler (Eppendorf), and the program consisted of 5 min at 22 °C, 30 min at 42 °C, and 5 min at 85 °C, finishing with a hold at 4 °C.

Cloning partial cDNA sequences for *cel, ctr2* and *pga2* from *R. quelen*

Degenerate primers (Table 1) were designed from the most highly conserved areas from sequences of cDNA between different fish species from GenBank for *carboxyl ester lipase* or *bile salt activated lipase* (*cel*) (*Ictalurus punctatus*, acc. no. XM_017493520.1; *Pygocentrus nattereri*, XM_017711355.1; *Astyanax mexicanus*, XM_007252785.2), *chymotrypsinogen 2* (*ctr2*) (*A. mexicanus*, XM_007246886.1, XM_007244272.2, XM_007244273.2, and XM_007246888.1) and *pepsinogen 2a* (*pga2*) (*I. punctatus*, NM_001201015.1; *I. furcatus*, GU588018.1; *P. nattereri*, XM_017722033.1 and XM_017687557.1; *Lates calcarifer*, XM_018666177.1), and were synthetized by IDT® (Integrated DNA Technologies, Leuven, Belgium). After cDNA synthesis, PCR amplifications were performed with the proofreading Q5® High-Fidelity DNA Polymerase (NewEngland BioLabs®*Inc.*). Then, reactions were cycled (98 °C, 5 min; [98 °C, 30 s; 65-55 °C in touchdown, 30 s; 72 °C, 1 min] x 35 cycles; and a final extension at 72 °C for 10 min) in a Mastercycler®proS vapo.protect thermal cycler (Eppendorf). PCR products were separated in 1 % agarose gels stained with GelRed, visualized under UV in a Gel Doc™ XR+ Gel Documentation System (BioRad). Bands were of the expected size and they were ligated into the pJET1.2/blunt Cloning Vector, using the CloneJET PCR Cloning Kit (Fermentas, Life Sciences), and used to transform *Escherichia coli* TOP10 strain, plated in LB ampicillin agar plates. Different colonies of each putative *cel*, *ctr2* and *pga2* partial cDNAs

were picked up, grown in liquid LB-ampicillin overnight at 37 °C, plasmid DNAs were extracted using the NucleoSpin® Plasmid kit (Macherey-Nagel) and then cDNAs were sequenced in both strands, using pJET1.2 forward and reverse sequencing primers by the dideoxy method (Stab Vida, Caparica, Portugal). The sequence homology for all the PCR products was confirmed by *blastn* from NCBI (Johnson et al., 2008) indicating the obtaining of partial clones for *cel, ctr2* and *pga2*.

Obtaining partial sequences for *pla2g1b* and *try2*, and full-length cDNA sequences for *anrt1a, anrt1b, anrt1l, anrt2, clock, per1, per2, per3, cry1a, cry1b, cry2, cry3, amy2a* and *ef1a* in *R. quelen*

The partial sequences of *phospholipase a2* secreted from pancreatic acinar cells in its inactive form (*pla2g1b*) and *trypsinogen 2* (*try2*) and the complete cDNA sequences of *aryl hydrocarbon receptor nuclear translocator-like 1a* (*arntl1a*), *aryl hydrocarbon receptor nuclear translocator-like 1b* (*arntl1b*), *aryl hydrocarbon receptor nuclear translocator-like 1-like* (*arntl1l*), *aryl hydrocarbon receptor nuclear translocator-like 2* (*arntl2*), *circadian locomotor output kaput* (*clock*), *period circadian regulator 1* (*per1*), *period circadian regulator 2* (*per2*), *period circadian regulator 3* (*per3*), *cryptochrome 1a* (*cry1a*), *cryptochrome 1b* (*cry1b*), *cryptochrome 2* (*cry2*), *cryptochrome 3* (*cry3*), *pancreatic α-amylase* (*amy2a*), *and elongation factor 1a* (*ef1a*) were obtained by RNA-seq analysis. The *β-actin* primers were used as previously described by Baldisserotto et al. (2014).

The RNA was extracted of three samples of kidney, brain + pituitary, and liver. The RNA quality was verified through TapeStation using RNA ScreenTape kit (Agilent) and Nanodrop (Thermo Scientific) and the concentration was measured with Qubit (Invitrogen). The mRNA capture and amplification were performed using Illumina TruSeq® Stranded mRNA HT in an Illumina NextSeq500 sequencer in 2x75 mode and the results were submitted to a quality inspection with Trimmomatic v0.36 program. Then the high-quality reads were aligned again with the Trinity v2.3.2 program. Due to the high number of contigs obtained, isoforms with abundance of less than 1 % in relation to all isoforms were excluded and, subsequently, a filtration was carried out at each level of abundance, maintaining only those with a TPM value higher than 1. Finally, redundant transcripts, i.e. with a similarity higher than 95 %, were eliminated through the CD-HIT-EST program and a functional annotation of the transcripts was performed through the Blast2GO software.

3'Rapid Amplification of cDNA Ends (RACE)

The 3' ends for *cel*, *ctr2, pga2*, *pla2g1b* and *try2* cDNAs were amplified from 1 µg of total RNA by 3' Rapid Amplification of cDNA Ends (RACE) using 3' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, ThermoFisher Scientific). Specific forward primers were designed in the five fragments previously cloned at two different positions and used in combination with the 3'AUAP or UAP primers supplied in the kit (Table 1).

5' Rapid Amplification of cDNA Ends (RACE)

The 5' ends for *cel*, *ctr2*, *pga2*, *pla2g1b* and *try2* cDNAs were amplified from 1 µg of total RNA by 5' Rapid Amplification of cDNA Ends (RACE) using FirstChoice® RLM-RACE kit (Life Technologies™, ThermoFisher Scientific). The specific reverse primers for each of five cDNAs (Table 2) were designed using Primer3 (v. 0.4.0), available at http://bioinfo.ut.ee/primer3-0.4.0/ (UNTERGASSER et al., 2012; KORESSAAR AND REMM, 2007), and used in combination with the 5' RACE Outer or Inner primers supplied in the kit. The primers were designed to achieve an overlap of at least 150 bp between the RACE clones and the previously obtained partial cDNAs. The cloning and sequencing of PCR products were performed as described above. For fragment assembly, the tool merger was used from EMBOSS Explorer graphical user interface (RICE, LONGDEN AND BLEASBY, 2000).

Quantification of mRNA expression levels

Specific primers used for qPCR analysis (Table 3) were designed using Primer3 software (v. 0.4.0) available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm. The optimization of qPCR conditions was made on primers annealing temperature (50 to 60 °C), primers concentration (200 and 400 nM) and template concentration (six different 1:10 dilution series from 10 ng to 100 fg of cDNA assumed from RNA input). Moreover, two negative controls, with i) 10 ng of RNA per reaction (NRT, non-reverse transcription control) and ii) sterile water (NTC, non-template control), were performed to detect possible gDNA contamination or primer-dimer artifacts. To perform qPCR reactions, 4 µL cDNA (10 ng assumed from RNA input), 0.5 µL of specific forward and reverse primers for each gene at the specified concentrations, and 5 µL of PerfeCta™ SYBR® Green Fastmix™ (Quanta BioSciences) were used in Hard-Shell® Low-Profile Skirted 96-Well PCR Plates sealed with Microseal® 'B' PCR Plate Adhesive Optical Sealing Film (Bio-Rad). Quantitative PCR was performed on a CFX Connect thermal cycler with CFX Manager™ Software (version 3.1; Bio-Rad Laboratories), and the thermal profile was as follows: 95 °C for 10 min; [denaturing at 95 °C for 15 s, annealing and extension at 60 °C for 30 s] x 40 cycles; melting curve from 60 to 95 °C increasing 0.5 °C

every 5 s. The melting curve was used to ensure that a single product was amplified and to check the absence of primer-dimer artifacts. Samples were run in triplicate and relative gene quantification was performed using the $\Delta\Delta C_T$ method (LIVAK AND SCHMITTGEN, 2001), corrected for efficiencies (Pfaffl, 2001) and using two internal reference genes (VANDESOMPELE et al., 2002), *actb* (larva: M = 0.269 and CV = 0.094; juveniles: M= 0.106 and CV= 0.307) and *ef1a* (larva: M = 0.269 and CV = 0.092; juveniles: M= 0.106 and CV= 0.307). The choice of these genes was due to their lower than 0.5 target stability M values and lower than 0.25 CVs, as indicated by BioRad CFX Manager Target Stability Value. Final primer concentration was 200 nM, except for *cry1a* that was 400 nM.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Homogeneity of variances among treatments was tested by Levene's test. Gene expression of clock and digestive enzymes genes comparisons along the 24 h period were assessed using one-way ANOVA and Tukey's post-hoc test. Data that did not exhibit homogeneous variances were assessed using non-parametric Kruskal-Wallis. The correlation between gene expression of clock genes and digestive enzymes genes was assessed using the Pearson Correlation. The analysis was performed using Statistica 7.0 (Stat Soft. Inc.) and SigmaPlot 11.0 softwares. The minimum significance level was set at P<0.05. The graphics were plotted in SigmaPlot 11.0 software.

Results

Expression of clock genes

In the post-larvae, *arntl1a* gene exhibited significant changes along the 24 h (p < 0.05) with the peak of expression at 1:00 h (1.07 ± 0.05) and the lower level expression at 17 h (0.63 ± 0.13) (Fig. 1A). The *clock* gene exhibited the same pattern observed in *arntl1a* gene expression. The peak of expression was at 1:00 h (1.33 ± 0.21), then it decreased to minimum levels at 17:00 h (0.41 ± 0.09) (Fig. 1E). The gene expression of *per1* showed a similar pattern to those observed in *arntl1a* and *clock*, but the maximum expression level began at 1:00 h (1.51 ± 0.31), remaining high until 9:00 h (1.28 ± 0.11) and then decreased to the minimum level at 17:00 h (0.36 ± 0.08) (Fig. 1F). With a similar pattern to that showed by the *per1* gene, the maximum gene expression of *per3* was detected at 1:00 h (1.56 ± 0.31), remaining until 5:00 h (1.25 ± 0.14) and then decreasing until the minimum level of expression at 17:00 h (0.46 ± 0.11) (Fig. 2B). The *per2* gene showed a different pattern of *per1* and *per3* genes, with maximum and minimum expression at 1:00 h (1.41 ± 0.29) and 17:00 h (0.66 ± 0.12),

respectively (Fig. 2A). The *cry2* gene exhibited a pattern slightly different from the other clock genes analyzed. The maximum level of gene expression was also at 1 h (1.92 ± 0.27), then decreased to the minimum level and remained constant at 17:00 h (0.79 ± 0.13) (Fig. 2E). The maximum expression of *cry3* was detected at 1:00 h (1.41 ± 0.18) and remaining at 5:00 h (1.17 ± 0.13), then decreased at minimum level at 17:00 h (0.46 ± 0.10) (Fig. 2F). The expression of the *arnt1b*, *arnt1l*, *arntl2*, *cry1a*, and *cry1b* genes did not exhibit significant changes in the post-larvae along the evaluated period (p > 0.05) (Figs. 1 and 2).

In the GIT of juveniles, *arntl1l* exhibited a peak of gene expression at 8:00 h (1.31 ± 0.53), decreasing and remained constant until the minimum level at 20:00 h (0.37 ± 0.07) (Fig. 3C). The gene expression of *cry2* showed a similar pattern to that observed in *arnt1l*, with the minimum level of gene expression also at 20:00 h (0.39 ± 0.03), but the maximum level was detected at 9:00 h (0.76 ± 0.10) and at 11:00 h (0.78 ± 0.08) (Fig. 4E). The expression of the *arntl1a*, *arntl1b*, *arntl2*, *clock*, *per1*, *per2*, *per3 cry1a*, *cry1b*, and *cry3* genes in the GIT of juveniles did not exhibit significant changes along the evaluated period (p > 0.05) (Figs. 3 and 4) .

Expression of digestive enzymes genes

In the post-larvae, *cel* gene showed the minimum level of expression at 1:00 h (0.81 ± 0.05), followed by an expression peak at 5:00 h (1.17 ± 0.04) and decreasing again to the minimum level at 9:00 h (0.80 ± 0.04) (Fig. 5B). The *pla2g1b* gene expression reached the maximum level at 5:00 h (1.11 ± 0.09) and the minimum level at 9:00 h (0.69 ± 0.03) (Fig. 5C). The *ctr2* gene showed the maximum level of gene expression at 5:00 h (1.34 ± 0.11) and the minimum level at 9:00 h (0.68 ± 0.03) (Fig. 5D). The *pga2* expression showed a different pattern, with the minimum level detected at 9:00 h (0.55 ± 0.05) followed by an increase up to the maximum level of expression, which was maintained throughout the evaluated period (Fig. 5F). The expression of the *amy2a* and *try2* genes in the post-larvae did not exhibit significant changes along the evaluated period (p > 0.05) (Fig. 5).

The digestive enzymes gene expression did not exhibit significant changes in the GIT of juveniles through the 24 h studied (p > 0.05) (Fig. 6).

Correlation between gene expression of clock genes and digestive enzymes genes

There was no correlation between the expression of the clock genes and the gene expression of the digestive enzymes in both post-larvae and GIT of juveniles (p > 0.05).

Discussion

The light/dark and feeding cycles are considered the strongest zeitgebers of the biological rhythms in animals because food is not constantly available in nature. However, due to the high plasticity of the circadian system, fish can anticipate the environmental changes, such as changes in food availability when exposed to a restricted feeding schedule (Mistlberger, 2009). In our study the rhythm of expression of the clock genes and the digestive enzymes genes were evaluated after feeding until the next meal time, for a period of approximately 24h and under LD photoperiod.

In silver catfish *R. quelen* post-larvae, most clock genes analyzed showed minimum level of expression in the light phase, while the maximum expression level occurred in the dark phase. The same pattern was found in the clock genes expression in GIT of juveniles, however only two genes showed differences during the evaluated period. Thus, the typical interaction between positive and negative feedback loops in *R. quelen* exposed to the LD cycle was not observed both in post-larvae and GIT juveniles, since the clock/arntl complex activates the *per* and *cry* transcription and high levels of per/cry dimer inhibits the clock/arntl complex, with maximum level *per* and *cry* expression associated with minimum level of *clock* and *arntl* expression (BELL-PEDERSEN et al., 2005; VATINE et al., 2011).

The results found for *R. quelen* post-larvae and juveniles contrast with those found in other species. In post-larvae, the positive (*arntl1a* and *clock*) and negative (*per1, 2, 3* and *cry 1, 2*) loop genes showed the acrophase at the same time. This is a different pattern from that found in gilthead seabream *Sparus aurata* larvae in different development stages submitted to LD cycle, which showed minimum gene expression levels of positive loop (*clock* and *bmal1*) when gene expression of negative loop (*cry* and *per3*) was maximum, at all stages evaluated (MATA-SOTRES et al., 2015). In the case of *R. quelen* juveniles, besides not presenting the typical interaction between the positive and negative loops, only one gene of positive loop (*arntl1l*) and one gene of negative loop (*cry2*) showed significant differences within 24 h, but one hour of difference between the expression peaks of this genes was observed. Contrasting with juveniles of gilthead seabream (VERA et al., 2013), Senegalese sole *Solea senegalensis* (MARTÍN-ROBLES et al., 2012) and goldfish *Carassius auratus* (FELICIANO et al., 2011) that presented the typical interaction between the positive and negative loops in liver. The difference between the expression of clock genes in juveniles of *R. quelen* and those from the other species mentioned can be explained by interspecific variation but also, probably, by the use of different tissues, since in our study the GIT was used and in the other studies the liver

was used. On the other hand, in studies with larvae the whole body is utilized, which minimizes the possibility of the difference between species be due to the use of different tissues.

Circadian rhythms can exert an important function in the control of the digestive system, and some digestive enzymes have circadian rhythmicity in their activity (SCHEVING et al., 2000). The post-larvae of *R. quelen* showed minimum level of gene expression of *pla2g1b*, *cel*, *ctr2,* and *pga2* one hour after feeding and maximum level of gene expression of these genes four hours before feeding. This result can be due to FAA, since the post-larvae were fed at the same time and kept under the same photoperiod through the acclimation period and 24 h of experiment. By restricting food at 8 h, gene expression increases as meal time approaches and transcription of genes from digestive enzymes increases (MISTLBERGER, 2009; PANDA, HOGENESCH AND KAY, 2002). Then, after the food offer RNA transcription decreases, avoiding a high energy cost that the continuous transcription would generate, since the body is already prepared for the digestive process (MATA-SOTRES et al., 2015). In *R. quelen* juveniles, the gene expression of digestive enzymes did not differ during the evaluated period. This can be explained, probably, by the presence of food throughout GIT through all sampling times, because is the time of emptying of the GIT that determines when the fish will feed again (YÚFERA et al., 2012). Likewise, the presence of the food in the GIT may have maintained the levels of gene expression of digestive enzymes constant due to the constant need of enzymatic action until the complete digestion of the food. The enzymes are constantly secreted (STECH, CARNEIRO AND PIZAURO JÚNIOR, 2009) and to supply this demand genes of these enzymes are probably transcribed and translated constantly.

Although there is no statistical relationship, there is a coincidence between the levels of expression of some clock genes and some digestive enzymes genes in post-larvae of *R. quelen*. The expression of clock genes reaches the highest value hours before the peak of digestive enzymes genes, with *per1* and *per3* maximum levels remaining until the peak of expression of the enzymatic genes. This pattern may indicate a probable modulation of the enzymatic expression by the clock genes, preparing the organism for the next feeding time, as occurs in larvae and juveniles of *S. aurata* (PAREDES et al., 2014; MATA-SOTRES et al., 2015, 2016).

In conclusion, *R. quelen* post-larvae and juveniles did not exhibit the typical interaction between positive and negative loop genes and only in post-larvae the gene expression of the digestive enzymes is possibly influenced by the clock genes. Finally, our results serve as the basis for conducting further studies, since there are no studies on daily gene expression of digestive enzymes and clock genes, as well as a possible correlation between them, in *R. quelen*.

References

ALMEIDA, A. P. G. et al. Composition of gastrointestinal content, preotease and lipase activities in summer and winter of four freshwater siluriforms (Teleostei: Actinopterygii) with two different feeding habits. **Zoologia**, v. 35, p. e13268, 2018.

BALDISSEROTTO, B. et al. The effects of ammonia and water hardness on the hormonal, osmoregulatory and metabolic responses of the freshwater silver catfish *Rhamdia quelen*. **Aquatic Toxicology**, v. 152, p. 341-351, 2014.

BELL-PEDERSEN, D. et al. Circadian rhythms from multiple oscillators: lessons from diverse organisms. **Nature Reviews Genetics**, v. 6, n. 7, p. 544-556, 2005.

DEL POZO, A. et al. Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass. **Physiology & Behavior**, v. 160, p. 446-450, 2012.

FELICIANO, A. et al. Feeding time synchronizes clock genes rhythmic expression in brain in liver of goldfish (*Carassius auratus*). **Journal of Biological Rhythms**, v. 26, v. 1, p. 24-33, 2011.

GOMES, L. C. et al. Biology of *Rhamdia quelen* (Teleostei, Pimelodidae). **Ciência Rural**, v. 30, n. 1, p. 179-185, 2001.

GOULART, F. R. et al. Atividade de enzimas digestivas e parâmetros de crescimento de juvenis de jundiá (*Rhamdia quelen*) alimentados com farelo de linhaça *in natura* e demucilada. **Semina: Ciências Agrárias**, v. 34, n. 6, p. 3069-3080, 2013.

JOHNSON, M. et al. NCBI BLAST: a better web interface. **Nucleic Acids Research**, v. 36, p. W5-W9, 2008.

KORESSAAR, T.; REMM, M. Enhancements and modifications of primer design program Primer3. **Bioinformatics**, v. 23, n. 10, p. 1289-1291, 2007.

KÜTTER, M. T.; BEMVENUTI, M. A.; MORESCO, A. Feeding strategy of the jundiá *Rhamdia quelen* (Siluriformes, Heptapteridae) in coastal lagoons of Southern Brazil. **Acta Scientiarum. Biological Sciences**, v. 31, n. 1, p. 41-47, 2009.

LAZZARI, R. et al. Protein source and digestive enzymes activities in jundiá (*Rhamdia quelen*). **Scientia Agricola**, v. 67, n. 3, p. 259-266, 2010.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2 - \Delta\Delta CT$ method. **Methods Enzymology**, v. 25, n. 4, p. 402-408, 2001.

LÓPEZ-OLMEDA, J. F. et al. Feeding entrainment of food-antecipatory activity and *per1* expression in the brain and liver of zebrafish under diferente lighting and feeding conditions. **Chronobiology International**, v. 27, n. 7, p. 1380-1400, 2010.

MARTÍN-ROBLES, A. J. et al. Cloning, tissue expression pattern and daily rhythms of *Period1*, *Period2* and *Clock* transcripts in the flatfish Senegalese sole, *Solea senegalensis*. **Journal of Comparative Physiology B**, v. 182, p. 673-685, 2012.

MATA-SOTRES, J. A. et al. Daily rhythms of clock gene expression and feeding behavior during the larval development in gilthead seabream, *Spaurus aurata*. **Chronobiology International**, v. 32, n. 8, p. 1061-1074, 2015.

MATA-SOTRES, J. A. et al. Daily rhythms of digestive enzyme activity and gene expression in gilthead seabream (*Spaurus aurata*) during ontogeny. **Comparative Biochemistry and Physiology, Part A**, v. 197, p. 43-51, 2016.

MISTLBERGER, R. E. Food-antecipatory circadian rhythms: concects and methods. **European Journal of Neuroscience**, v. 30, n. 9, p. 1718-1729, 2009.

MONTOYA, A. et al. Feeding time synchronises daily rhythms of behavior and digestive physiology in gilthead seabream (*Sparus aurata*). **Aquaculture**, v. 306, p. 315-321, 2010.

MORGAN, E. Ecological significance of biological clocks. **Biological Rhythms Research**, v. 35, n. 1-2, p. 3-12, 2004.

MORO, G. V. Dietary non-protein energy source: growth, digestive enzymes activities and nutriente utilization by the catfish jundiá, *Rhamdia quelen*. **Aquaculture Research**, v. 41, n. 3, p. 1-5, 2010.

PANDA, S.; HOGENESCH J. B.; KAY, S. A. Circadian rhythms from flies to human. **Nature**, v. 417, p. 329-335, 2002.

PFAFFL, M. W. A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Research**, v. 29, n. 9, p. 2002-2007, 2001.

PAREDES, J. F. et al. Circadian rhythms of gene expression of lipid metabolism in Gilthead Sea bream liver: Synchronisation to light and feeding time. **Chronobiology International**, v. 31, n. 5, p. 613-626, 2014.

RICE, P.; LONGDEN, I.; BLEASBY, A. EMBOSS: The European Molecular Biology Open Software Suite. **Trends in Genetics**, v. 16, n. 6, p. 276-277, 2000.

SÁNCHEZ, J. A.; SÁNCHEZ-VÁZQUEZ, F. J. Feeding entrainment of daily rhythms of locomotor activity and clock gene expression in zebrafish brain. **Chronobiology International**, v. 26, p. 1120-1135, 2009.

SCAGLIONE, M. C. Light and dark rations and the photic entrainment of circadian locomotor activity patterns in the South American Silver Catfish (*Rhamdia quelen*, Quoy E Gaimard, 1824). **Biological Rhythm Research**, v. 49, n. 1, p. 129-140, 2018.

SCHEVING, L. A. Biological Clocks and the Digestive System. **Gastroenterology**, v. 119, p. 536-549, 2000.

SCHULZ, U. H.; LEUCHTENBERGER, C. Activity patterns of South American silver catfish (*Rhamdia quelen*). **Brazilian Journal of Biology**, v. 66, n. 2, p. 565-574, 2006.

STECH, M. R.; CARNEIRO, D. J.; PIZAURO JÚNIOR, J. M. Fatores que afetam a produção de enzimas digestivas em peixes e o uso de enzimas exógenas como ferramentas em nutrição de peixes. **Ensaios e Ciência**, v. 13, n. 2, p. 79-93, 2009.

STEPHAN, F. K. The "Other" Circadian System: Food as a Zeitgeber. **Journal of Biological Rhythms**, v. 17, n. 4, p. 284-292, 2002.

UNTERGASSER, A. et al. Primer3-new capabilities and interfaces. **Nucleic Acids Research**, v. 40, n. 15, p. e115, 2012.

VANDESOMPELE, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biology**, v. 3, n. 7, p. Research0034.1-Research0034.11, 2002.

VATINE, G. et al. It's time to swin! Zebrafish and the circadian clock. **FEBSLetters**, v. 585, n. 10, p. 1485-1494, 2011.

VERA, L. M. et al. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. **Physiology & Behavior**, v. 90, n. 2-3, p. 518-524, 2007.

VERA, L. M. et al. Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Spaurus aurata*). **Cronobiology International**, v. 30, n. 5, p. 649-661, 2013.

YÚFERA, M. et al. Acidic Digestion in a Teleost: Postprandial and Circadian Pattern of Gastric pH, Pepsin Activity, and Pepsinogen and Proton Pump mRNAs Expression. **PlosOne**, v. 7, n. 3, p. e33687, 2012.

Figure 1 – Relative expression of *arntl1a* (A), *arntl1b* (B), *arntl1l* (C), *arntl2* (D), *clock* (E) and *per1* (F) genes in post-larvae of *Rhamdia quelen*. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
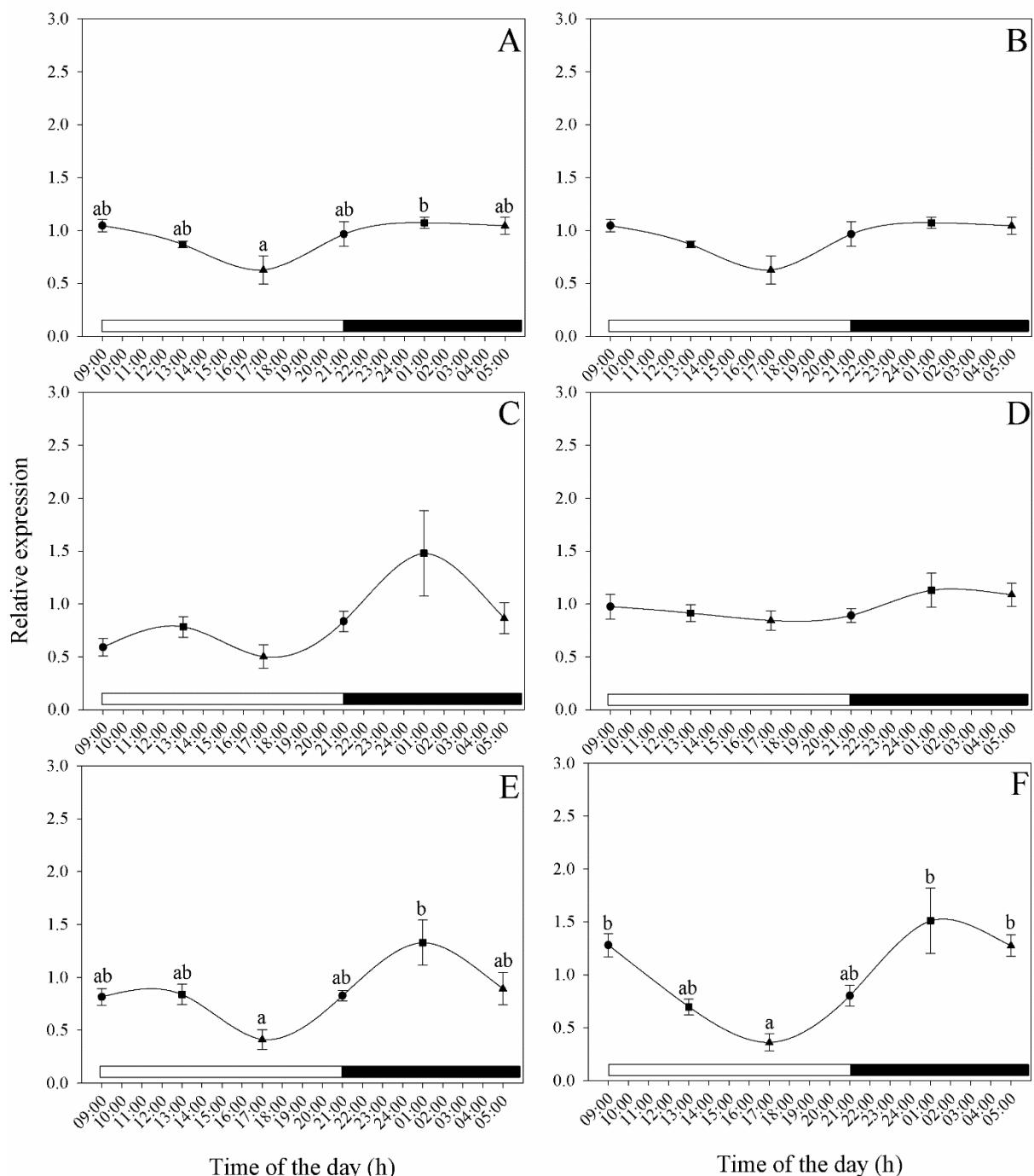
Figure 2 – Relative expression of *per2* (A), *per3* (B), *cry1a* (C), *cry1b* (D), *cry2* (E) and *cry3* (F) genes in post-larvae of *Rhamdia quelen*. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
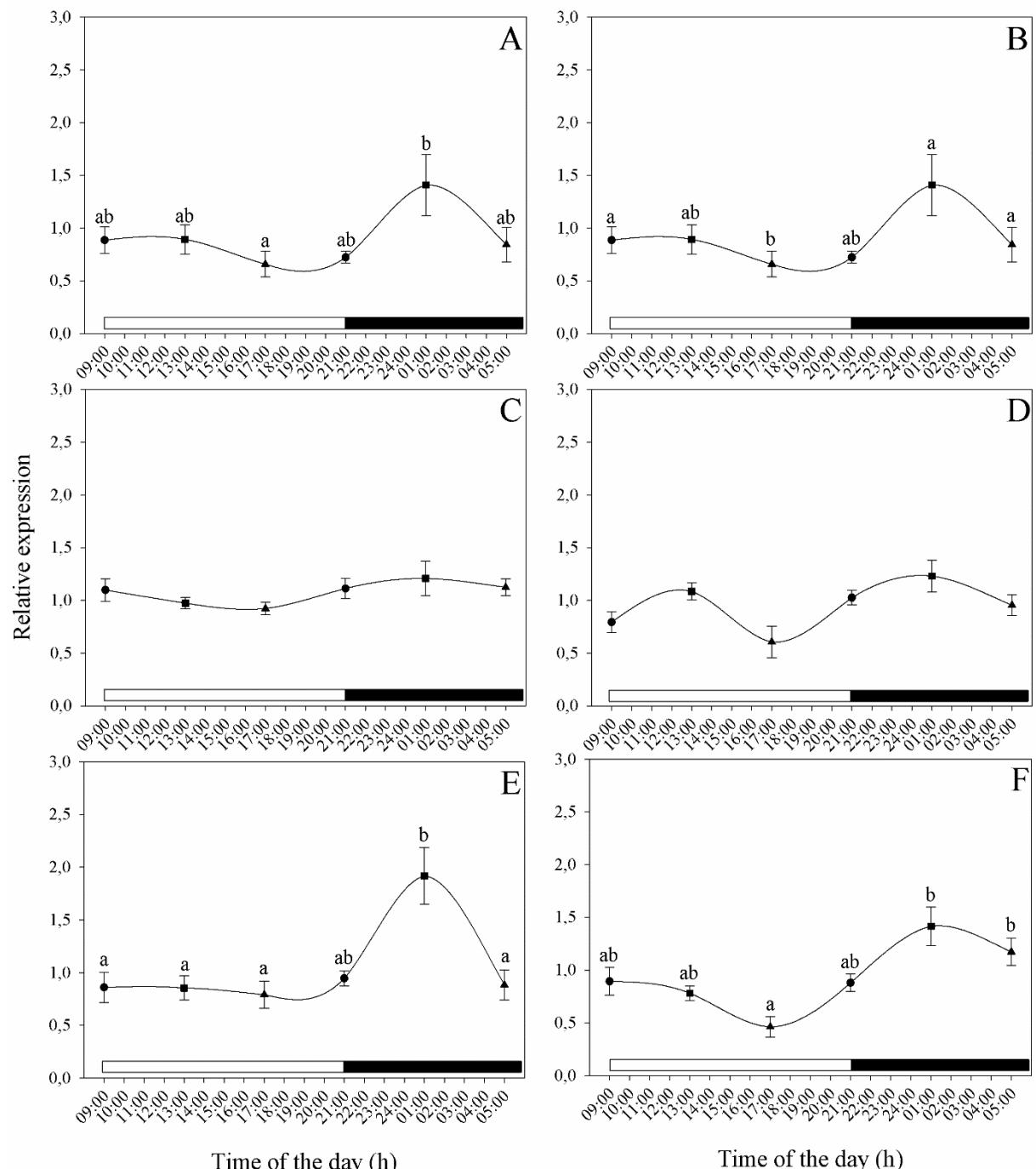
Figure 3 – Relative expression of *arntl1a* (A), *arntl1b* (B), *arntl1l* (C), *arntl2* (D), *clock* (E) and *per1* (F) genes in gastrointestinal tract of *Rhamdia quelen* juveniles. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
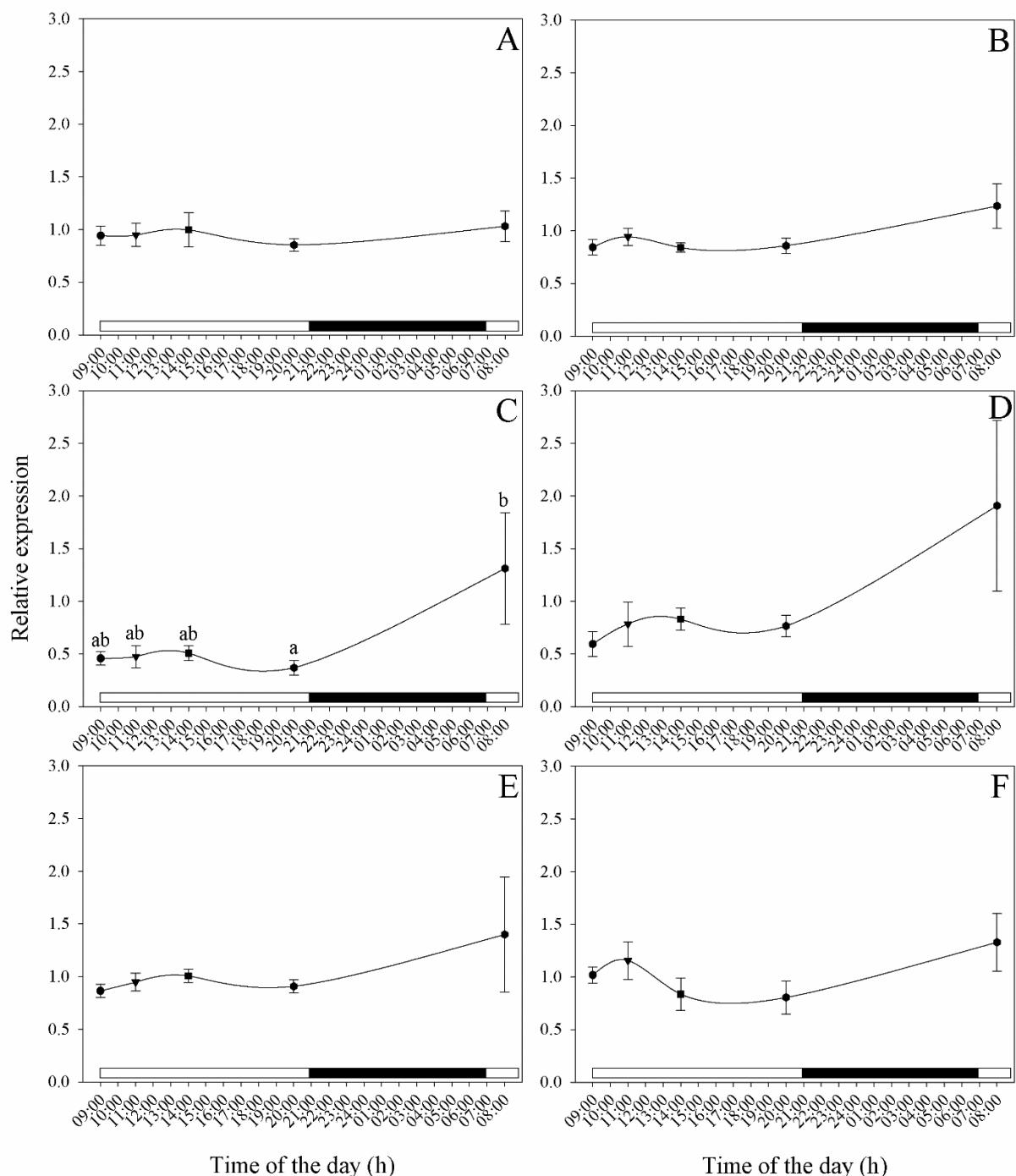
Figure 4 – Relative expression of *per2* (A), *per3* (B), *cry1a* (C), *cry1b* (D), *cry2* (E) and *cry3* (F) genes in gastrointestinal tract of *Rhamdia quelen* juveniles. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
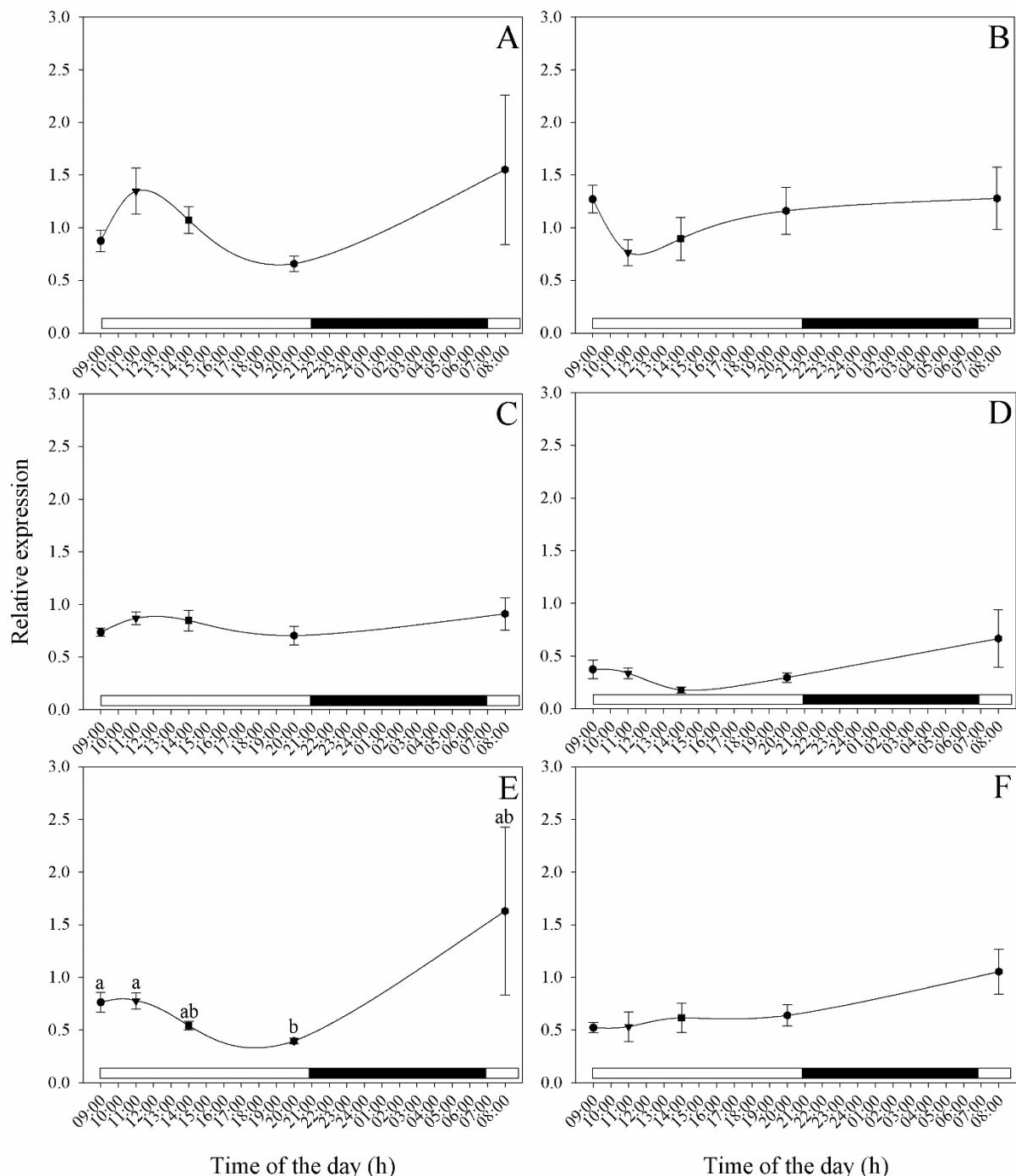
Figure 5 – Relative expression of *amy2a* (A), *cel* (B), *pla2g1b* (C), *ctr2* (D), *try2* (E) and *pga2* (F) genes in post-larvae of *Rhamdia quelen*. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
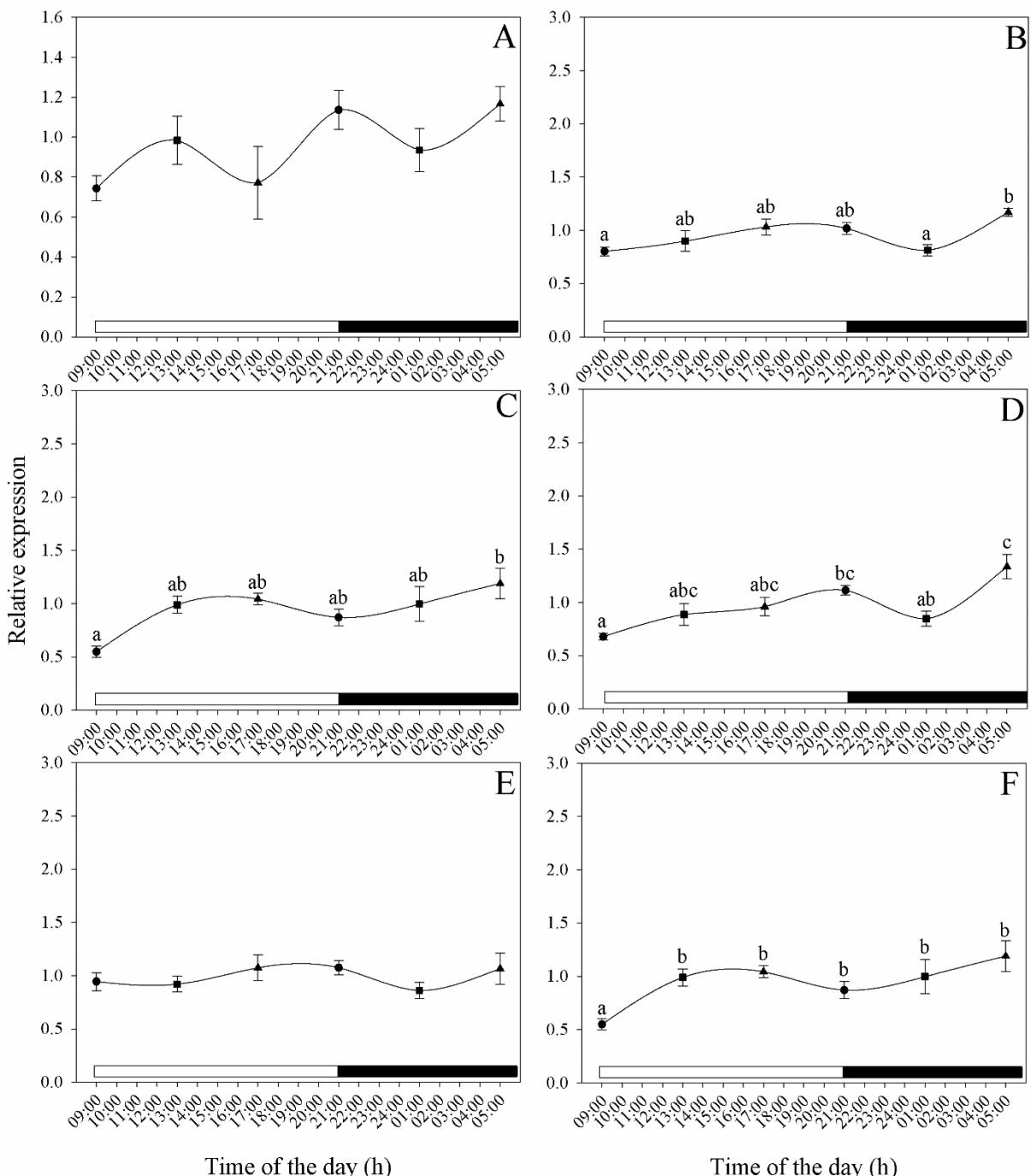
Figure 6 – Relative expression of *amy2a* (A), *cel* (B), *pla2g1b* (C), *ctr2* (D), *try2* (E) and *pga2* (F) genes in *Rhamdia quelen* juveniles. The white and black bars at down of the graphs indicate the light and dark periods, respectively. Date are shown as the mean ± SEM. Different letters indicate significant differences based on ANOVA one-way and Tukey's Test (p<0.05).
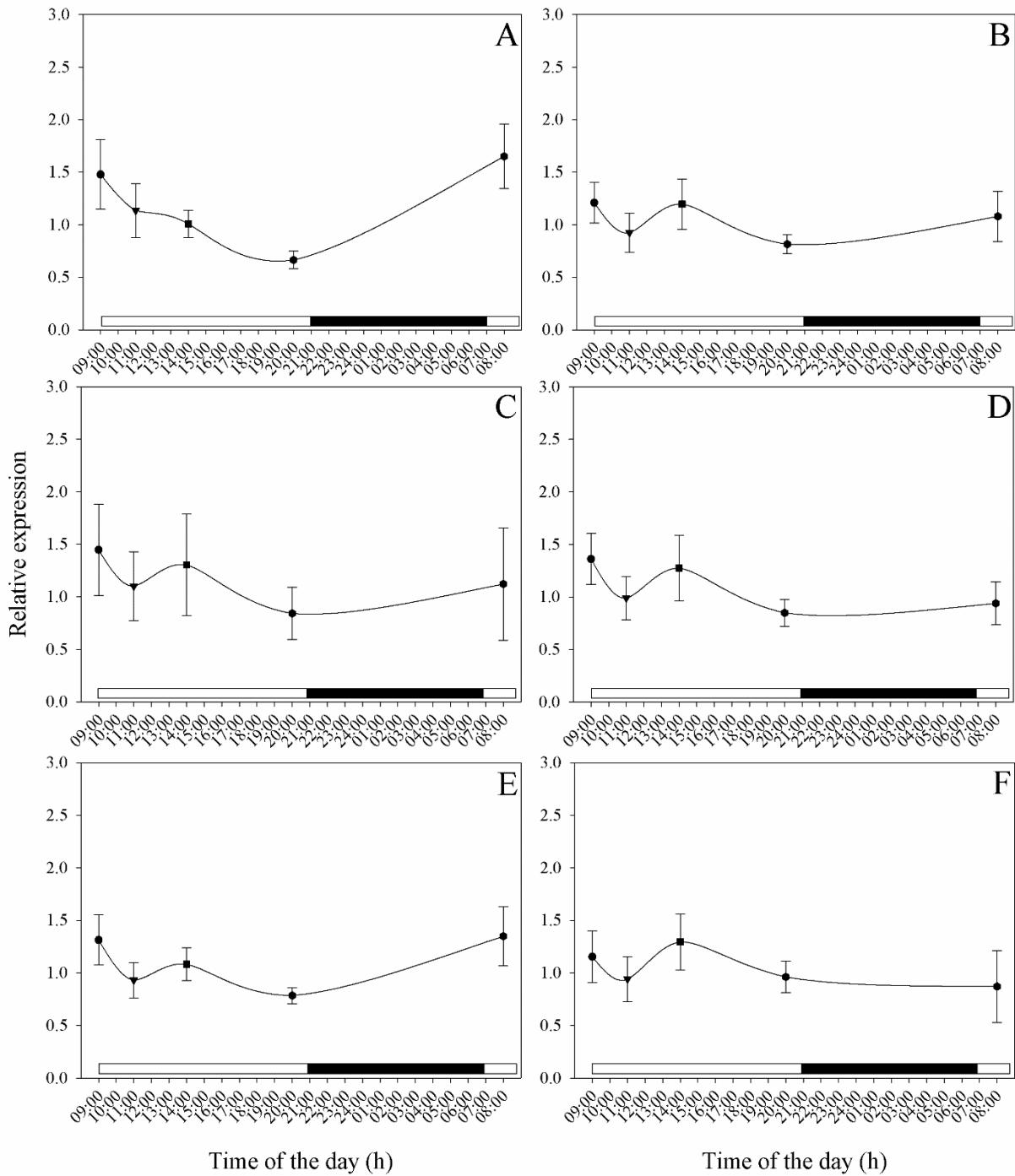
Table 1 – Primers designed for molecular identification of partial cDNAs for *cel*, *ctr2* and *pga2*, as well as primers used for 3' and 5' Rapid Amplification cDNA Ends (RACE), in addition to *pla2g1b* and *try2*.

Primers	Nucleotide sequence 5' → 3'	Position	Size amplified
Degenerate			
RqCEL_F1	TGGYTGKGYTRGGRATTTT	53	1,222
RqCEL_R1	GRTRTARTCAGTCTCRATKTCCAC	1,274	
RqCEL_F2	ATGCAKCYWCKYTGGGAG	104	963
RqCEL_R2	ATGTGRCCRTCCATGTYRTTRACT	1,066	
RqCTR2_F1	DMCARRATTGTGAAYGGTG	104	566
RqCTR2_R1	RCASACCAGRGGRCCRCCA	669	
RqPGA2_F1	AGMAAGGMYTKTGGGAGR	136	853
RqPGA2_R1	YCDTTRAGRGTGAAGGTR	988	
3' RACE			
CEL 3'outer	CTATCTTTGGTGAGTCTGCCG	668	1,137
CEL 3' inner	ATTTCCCAGAGTGGTGTTGC	751	1,054
CTR2 3'outer	CTGTGTGTTTGGCTGAGACC	425	471
CTR2 3' inner	GCAAACACTCCTGCTCGTCT	511	385
PGA2 3'outer	TGAGGTGCTGTTTGGTGAGA	686	739
PGA2 3' inner	TGGACAGCGTTACTATCAATGG	778	647
PLA2 3' outer	GCATGACCAGTGCTACAGTGA	255	349
PLA2 3' inner	TGCTGAGTGCTTCTCTGTGTC	426	178
TRY 3' outer	GGTTATGGATGTGCTGAGAAGA	513	194
TRY 3' inner	CATCCTGGTGTCTATGCCAA	537	170
5' RACE			
CEL 5'outer	ACTGAGAAATCCGAGCGTCC	558	558
CEL 5' middle	CGCTTCCACGAGTTCCAC	337	337
CEL 5' inner	TCTGGAATCTGCCTGGTTGT	235	235
CTR2 5'outer	GGTCTCAGCCAAACACACAG	444	444
CTR2 5' middle	ATTTCTCGTGCCTGATGGAC	346	346
CTR2 5' inner	AGCAGCAGTTACCACCCAGT	234	234
PGA2 5'outer	TCTCACCAAACAGCACCTCA	705	705
PGA2 5' middle	GAGATTCCACCAACCGACAC	484	484
PGA2 5' inner	TGTTGCTCTGGAAAGTGCTG	402	402
PLA2G1B 5'outer	GACACAGAGAAGCACTCAGCA	446	446
PLA2G1B 5' middle	CAGTGTATGGGTTGTCCAGG	325	325
PLA2G1B 5' inner	GGCTGAACACAGACGATCAT	155	155
TRY2 5'outer	TTGGCATAGACACCAGGATG	556	>556
TRY2 5' middle	TCTTCTCAGCACATCCATAACC	534	>534
TRY2 5' inner	ATGAGTCCTTGCCTCCCT	450	>450

Table 2 – Specific primers used for real-time PCR.

Primers	Direction	Nucleotide sequence 5' → 3'	Position	Amplicon Size (bp)	Larvae		GIT		Accession number
					E (%)	R²	E (%)	R²	
rqQactb	Forward	ATTGGCAATGAGAGGTTCAGG	847	122	91.05	0.999	97.96	0.999	KC195970.1
	Reverse	CGGATATCGACGTCACACTTC	968						
rqQef1a	Forward	AAACCTGGGCACTCTACTCAA	1,511	100	92.62	0.999	97.96	0.999	
	Reverse	GGTCAAACAATGAAGCCACA	1,610						
rqQanrt1a	Forward	CCACCGCACAGAGGTAGC	2,623	112	98.98	0.999	94.08	0.996	
	Reverse	ATTGAAACGCCAACAGTGAC	2,734						
rqQanrt1b	Forward	GCTGCTGGATGGAGGAACAT	3,669	126	98.43	0.999	101.5	0.998	
	Reverse	AGGTAAGGAGAGCGGAAGGA	3,794						
rqQanrt1l	Forward	AGGCCAGGACTCCATAGGTT	1,887	126	92.27	0.999	102.5	0.999	
	Reverse	ATGGCTGCCTCATCGTTACT	2,012						
rqQanrt2	Forward	TGCTGGAACCCTTCGATATT	3,633	127	100.35	0.998	95.28	0.993	
	Reverse	GAGCCCGGAGTAAGAACAGG	3,759						
rqQclock	Forward	AAGCGGTGTTTATTTACGGTTC	2,745	112	96.99	0.996	97.16	0.992	
	Reverse	CACGGTTATTGCTGCGAGAT	2,856						
rqQper1	Forward	GCTGCTTATGGCTGACACTG	4,233	129	100.01	0.997	95.1	0.999	
	Reverse	GTAAACCGAGGCTGATGCTT	4,361						
rqQper2	Forward	GAGGCATGTGTTGGCTGTAA	4,315	119	94.33	0.999	109.46	0.996	
	Reverse	TTCGTCTGGTCATGTGTTGG	4,433						
rqQper3	Forward	GTTCTCTGGGTTCGGGTTC	3,617	105	101.15	0.999	94.56	0.994	
	Reverse	TGTCTGAGGAGTCGTTGCTG	3,721						

Table 2. Continued

Primers	Direction	Nucleotide sequence 5' → 3'	Position	Amplicon Size (bp)	Larvae E (%)	Larvae R²	GIT E (%)	GIT R²	Accession number
rqQcry1a	Forward	CATAGAGCGCATGAAGCAAA	1,473	145	90.51	0.990	102.48	0.996	
	Reverse	GTCCTGGGTGGCATCTGTAG	1,617						
rqQcry1b	Forward	GGGCAATCCCATCTGTGT	1,140	129	95.96	0.997	98.52	0.998	
	Reverse	ATCCAACCCTCCTGTCTCAG	1,268						
rqQcry2	Forward	CGTAACCCAGAGGCATTAGC	1,063	135	94.08	0.996	104.69	0.995	
	Reverse	GGTAAGGAAACATGCAACAGC	1,197						
rqQcry3	Forward	AACGGACCCTGACCCTAATC	1,770	144	99.30	0.993	103.55	0.998	
	Reverse	TTACTCTGGCTGGTGTGCTG	1,913						
rqQamy2a	Forward	AACGGGCGTGCTTACTTTA	1,502	114	98.13	0.998	91.67	0.999	
	Reverse	TGGCTGGTGCTCATCATTTA	1,615						
rqQcel	Forward	CACTCCACTGGGCTACTTCC	1,443	80	94.84	0.995	94.62	0.999	
	Reverse	CAGTCTTGGCGAAGTTGGTC	1,522						
rqQpla2g1b	Forward	ATCTGTGAATGTGACCGGAAG	423	80	96.37	0.997	100.62	0.998	
	Reverse	TCACTGGAGACGCTCTTGTTT	482						
rqQctr2	Forward	CTGTGTGTTTGGCTGAGACC	425	105	96.40	0.999	98.51	1.000	
	Reverse	GACGAGCAGGAGTGTTTGC	529						
rqQtry2	Forward	CCTGGTGTCTATGCCAAGGT	540	93	94.90	0.998	94.70	0.999	
	Reverse	GATGTTGCATTGTGAACTGGA	632						
rqQpga2	Forward	CCTCCTTCATCCTATGTCAGC	1,005	136	92.15	0.996	96.75	0.996	
	Reverse	CACTGTTGGTTTGCCTGTTG	1,140						

4 DISCUSSÃO GERAL

Na aquicultura os peixes são submetidos a situações estressantes inerentes a atividade como biometria, vacinação, tratamentos, cirurgias, extrusão de gametas, marcação, amostragem de material biológico para análises patológicas, altas densidades de estocagem e transporte. A exposição aguda ou crônica a essas práticas leva a perda de homeostasia, o qual podemos denominar estresse (SUMMERFELT e SMITH, 1990; COYLE, DURBOROW e TIDWELL, 2004). A resposta ao estresse consiste na ativação simpática do eixo HII que desencadeará eventos fisiológicos com o intuito de auxiliar o animal a reagir e superar tal situação, garantindo sua sobrevivência (BARTON e IWAMA, 1991). Com o propósito de amenizar a ativação do eixo HII durante as práticas na aquicultura, substâncias com efeitos sedativos e anestésicos tem sido utilizadas (COOKE et al., 2004; INOUE et al., 2005). No entanto, estudos demonstram que a exposição a anestésicos sintéticos pode causa efeitos adversos (CARTER, WOODLEY e BROWN, 2011; GOMES et al., 2011; ZAHL et al., 2011; SNEDDON, 2012) incentivando a pesquisa por alternativas naturais como os óleos essenciais extraídos de plantas.

As espécies vegetais *Aloysia triphylla* e *Lippia alba* apresentam relatos sobre o uso do chá de suas folhas na medicina popular devido a suas propriedades analgésicas e sedativas (LORENZI e MATOS, 2008; EL-HAWARY et al., 2012; PASCUAL et al., 2001). A eficácia anestésica dos OEs dessas plantas já foi comprovada em várias espécies de animais aquáticos (CUNHA et al., 2010, 2011; TONI et al., 2013; GRESSLER et al., 2014; PARODI et al., 2012, 2014; ZEPPENFELD et al., 2014; BECKER et al., 2015; CÁRDENAS et al., 2016; TEIXEIRA et al., 2017).

No presente estudo foi comprovada a eficácia anestésica dos óleos essenciais de *A. triphylla* e de L*. alba* em *Serrasalmus rhombeus* e *S. eigenmanni*, duas espécies amazônicas habitantes do Rio Negro. Foi constatado que *S. rhombeus* é mais resistente ao efeitos anestésico de ambos OEs quando comparado com *S. eigenmanni*. No artigo 1, as concentrações indicadas para anestesia profunda (estágio 3) em *S. rhombeus* é de 150 e 200 µL L^{-1} dos OEs de *A. triphylla* e de *L. alba*, respectivamente. Ao passo que no manuscrito 2, observamos que a concentração indicada para anestesia profunda em *S. eigenmanni* é de 100 µL L^{-1} de ambos OEs. Para a sedação, foi comprovado que a concentração de 50 µL L^{-1} de ambos os óleos foi suficiente para sedar *S. rhombeus*,

enquanto que *S. eigenmanni* necessitou de 25 e 50 µL L^{-1} dos OEs de *A. triphylla* e *L. alba*, respectivamente, para atingir o estágio de sedação.

Nos experimentos realizados para avaliar a viabilidade do uso de ambos os OEs em estudos de transporte, foi comprovado que ambos os OEs podem ser utilizados. Para o transporte de peixes, se recomenda a utilização de concentrações baixas que somente sedem os animais, diminuindo a resposta a estímulos externos e a taxa metabólica, mas sem causar perda de equilíbrio (SUMMWERFELT e SMITH, 1990; PIRHONEN e SCHRECK, 2003). No artigo 1, verificamos que as concentrações de 5 e 10 µL L^{-1} de ambos OEs foram eficientes na redução da atividade natatória de *S. rhombeus* expostos durante 15 min a essas substâncias, sem perda de equilíbrio do animal. No entanto, para o OE essencial de *A. triphylla* é recomendada a concentração de 5 µL L^{-1} por ter apresentado menor tempo de natação. O mesmo foi observado no manuscrito 2, ambos os OEs reduziram a atividade natatória de *S. eigenmanni* expostos durante 4 horas. Contudo, os peixes expostos a concentração de 10 µL L^{-1} do OE de *L. alba* perderam o equilíbrio após 1 hora de exposição, portanto, não é recomendada para o transporte pois nesse estágio o peixe pode morrer por sufocamento (COOKE et al., 2014). Os peixes expostos a concentração de 5 µL L^{-1} de *A. triphylla* mantiveram o equilíbrio durante as 4 horas de exposição e apresentaram uma diminuição na excreção de amônia, bem como no tempo de natação.

Ritmos biológicos são caracterizados como eventos que ocorrem periodicamente em um organismo, sendo os mais explorados os ritmos associados aos ciclos geofísicos que permitem a adaptação do animal (MORGAN, 2004). Os organismos, durante o processo evolutivo, desenvolveram estratégicas comportamentais e fisiológicas que os permitem adaptar suas atividades antecipadamente e, com isso, otimizar seus processos biológicos (VERA et al., 2009). Os estímulos ambientais atuam como sincronizadores externos, ou *zeitgebers*, do ritmo biológico, sendo que o sincronizador mais poderoso é o ciclo claro/escuro ambiental (VERA et al., 2007). Entretanto, existem outras variáveis ambientais que podem atuar como sincronizadores dos ritmos biológicos, por exemplo a disponibilidade de alimento (ZHDANOVA & REEBS, 2006). Nos peixes, o ritmo circadiano está sob o controle da glândula pineal que apresenta fotossensibilidade e possui um relógio intrínseco responsável pela produção e secreção do hormônio melatonina e pela expressão de genes relógio (FÁLCON et al., 2010; NOCHE et al., 2010).O controle molecular do ritmo circadiano em peixes é baseado na interação entre alças de retroalimentação transcricional-translacional de alças de genes relógio positiva (*clock* e

bmal) e negativa (*per* e *cry*), sendo semelhante aos demais organismos (CYMBOROWSKI, 2010). De modo geral, é esperado que a expressão gênica máxima dos genes *clock* e *bmal* ocorra quando a expressão gênica de *per* e *cry* sej a mínima. No entanto, nesse estudo verificamos, no manuscrito 3, que em larvas e no trato gastrintestinal (TGI) de juvenis, ambos expostos a um fotoperíodo 14:10 LD, isso não ocorre. Tanto os genes das alça positiva quanto os genes da alça negativa apresentaram maior expressão gênica e menor expressão gênica nos mesmos horários. Isso pode ser explicado por variações entre espécies mas também, no caso dos juvenis, provavelmente, pela utilização do TGI nessse estudo pois a maioria dos estudos utilizam o fígado, como oscilador periférico, para quantificar a expressão gênica dos genes relógios (FELICIANO et al., 2011; MARTÍN-ROBLE et al., 2012; VERA et al., 2013).

O ritmo circadiano também pode exercer um papel importante no controle da digestão e as enzimas digestivas (SCHEVING et al., 2000). Constatamos que, em larvas, a expressão gênica das enzimas digestivas apresentou maiores níveis 4 h antes da alimentação e menores níveis 1 h depois da alimentação. Isso, provavelmente, pode ser explicado como uma preparação para alimentação, uma vez que as larvas foram alimentadas diariamente no mesmo horário. Com a aproximação do horário da alimentação, a transcrição e a expressão gênica aumentam (MISTLBERGER, 2009; PANDA, HOGENESCH AND KAY, 2002). Já no TGI de juvenis, não foram detectadas diferenças nos níveis de expressão gênica das enzimas digestivas. No entanto, foi detectada a presença de alimento em todo o TGI de todos os animais em todos os horários de coleta, o que pode ter influenciado na manutenção de níveis constantes de expressão gênica das enzimas para que o peixe possa digerir completamente o alimento. Sabe-se que o tempo de esvaziamento do TGI que determina quando o animal irá ingerir alimento novamente (YÚFERA et al., 2012). Observou-se em larvas que, embora não tenha sido constatada uma correleção estatística, existem um padrão na expressão gênica dos genes relógios quando comparados a expressão gênica das enzimas digestivas. Esse padrão pode indicar uma possível modulação da expressão gênica das enzimas digestivas pelos genes relógio preparando o organismo para a próxima alimentação como em larvas e juvenis de *Sparus aurata* (PAREDES et al., 2014; MATA-SOTRES et al., 2015, 2016).

5 CONCLUSÕES GERAIS

- As concentrações indicadas para anestesia em *S. rhombeus* são 150, 200 e 50 µL L^{-1} de OE de *A. triphylla*, OE de *L. alba* e do eugenol, respectivamente.

- As concentrações indicadas para sedação em *S. rhombeus* são 50, 50 e 25 µL L^{-1} de OE de *A. triphylla*, OE de *L. alba* e do eugenol, respectivamente.

- Para estudos de transportes com *S. rhombeus* são indicadas as concentrações de 5 µL L^{-1} de OE de *A. triphylla*, 5 e 10 µL L^{-1} de OE de *L. alba* e 10 µL L^{-1} do eugenol.

- A concentração indicada para anestesia em *S. eigenmanii* é de 100 µL L^{-1} de OE de *A. triphylla* e do OE de *L. alba*.

- As concentrações indicadas para sedação em *S. eigenmanii* é de 25 e 50 µL L^{-1} de OE de *A. triphylla* e do OE de *L. alba*, respectivamente.

- A concentração de 5 µL L^{-1} de OE de *A. triphylla* é recomendada para transporte de até 4 h de *S. eigenmanni*.

- Pós-larvas e juvenis de *R. quelen* expostos a um fotoperíodo 14:10 LD não apresentam a interação típica esperada entre as alças positiva e negativa de genes relógio.

- Embora não haja uma correlação estatística, o padrão observado sugere uma possível modulação da expressão gênica das enzimas digestivas pelos genes relógios em pós-larvas de *R. quelen*.

- A presença de alimento no TGI dos juvenis de *R. quelen*, pode ter interferido na expressão gênica das enzimas digestivas, que se manteve constante durante o período avaliado, sugerindo uma digestão lenta.

REFERÊNCIAS

BAKER, M. R.; GOBUSH, K. S.; VYNNE, C. H. Review of factors influencing stress hormones in fish and wildlife. **Journal for Nature Conservation**, v. 21, p. 309-318, 2013.

BAKKALI, F. et al. Biological effects of essential oils – A review. **Food and Chemical Toxicology**, v. 46, p. 446-475, 2008.

BARBAS et al. Properties of two plant extractives as anaesthetics and antioxidants for juvenile tambaqui *Colossoma macropomum*. **Aquaculture**, v. 469, p. 79-87, 2017.

BARTON, B. A.; IWANA, G. K. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. **Annual Review of Fish Diseases**, v. 10, p. 3-26, 1991.

BARTON, B. A. Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. **North American Journal of Aquaculture**, v. 62, p. 12-18, 2000.

BARTON, B. A. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. **Integrative and Comparative Biology**, v. 42, p. 517-525, 2002.

BECKER, et al. Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and essential oil of *Lippia alba*. **Fish Physiology and Biochemistry**, v. 38, n. 3, p. 789-796, 2012.

BENOVIT, S. C. et al. Anesthesia and transport of Brazilian flounder, *Paralichthys orbignyanus*, with essential oils of *Aloysia gratissima* and *Ocimum gratissimum*. **Journal of the World Aquaculture Society**, v. 43, p. 896-900, 2012.

BIANCHINI et al. Monoterpenoids (thymol, carvacrol, and S-(+)linalool) with anesthetic activity in silver catfish (*Rhamdia quelen*): evaluation of acetilcholinesterase and GABAergic activity. **Brazilian Journal of Medical and Biological Research**, 50:e6346, 2017.

BIASI, L. A.; COSTA, G. Propagação vegetativa de *Lippia alba*. **Ciência Rural**, v. 33, p. 455-459, 2003.

BOCKMANN, F. A. Família Heptapteridae. In: BUCKUP, P. M.; MENEZES, N. A.; GHAZZI, M. S. (Editores). **Catálogo das espécies de peixes de água doce do Brasil**. Rio de Janeiro: Museu Nacional, 2007, p. 104-109.

BOLLIET, V.; ARANDA, A.; BOUJARD, T. Demand-feeding rhythm in rainbow trout and European catfish. Synchronisation by photoperiod and food availability. **Physiology & Behavior**, v. 73, n. 4, p. 625-633, 2001.

BUCKUP, P. A.; TEIXEIRA, J. M. S. Família Cichlidae. IN: BUCKUP, P. M.; MENEZES, N. A.; GHAZZI, M. S. (Editores). **Catálogo das espécies de peixes de água doce do Brasil**. Rio de Janeiro: Museu Nacional, 2007, p. 139-149.

BOLASINA, S. N. Stress response of juvenile flounder (*Paralichthys orbignyanus*, Valenciennes 1839), to acute and chronic stressors. **Aquaculture**, v. 313, n. 1, p. 140-143, 2011.

CÁRDENAS et al. Effects of clove oil, essential oil of *Lippia alba* and 2-phe anaesthesia on juvenile meagre, *Argyrosomus regius* (Asso, 1801). **Journal of Applied Ichthyology**, v. 32, n. 4, 2016.

CARSON, C. F.; HAMMER, K. A.; RILEY, T. V. *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. **Clinical and Microbiological Review**, v. 19, p. 50-62, 2006.

CARTER, K. M.; WOODLEY, C. M.; BROWN, R. S. A review of tricaine methanessulfonate for anesthesia of fish. **Fish Biology and Fisheries**, v. 21, n. 1, p. 51-59, 2006.

CECON, E.; FLÔRES, D. E. F. L. Regulação da expressão gênica nas engrenagens do relógio circadiano em mamíferos. **Revista da Biologia**, v. 4, p. 28-33, 2010.

CHAIEB, K. et al. The chemical composition and biological activity of clove essential, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. **Phytotherapy Research**, v. 21, n. 6, p. 501-506, 2007.

CHRUBASIK, S.; PITTLER, M. H.; ROUFOGALIS, B. D. A comprehensive review on the ginger effect and efficacy profiles. **Phytomedicine**, v. 12, p. 684-701, 2005.

COOKE, S. J. et al. Behavioral and physiological assessment of low concentrations of the clove oil anaesthetic for handling and transporting largemouth bass (*Micropterus salmoides*). **Aquaculture**, v. 239, p. 509-529, 2004.

COYLE, S. D.; DURBOROW, R. M.; TIDWELL, J. H. Anesthetic in aquaculture. **Southern Regional Aquaculture Center (SRAC)**, Publication 3900, 2004.

COWAN, M.; AZPELETA, C.; LÓPEZ-OLMEDA, J. F. Rhythms in the endocrine system of fish: a review. **Journal of Comparative Physiology B**, n. 187, v. 8, p.1057-1089, 2017.

CUNHA, M. A. et al. Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. **Ciência Rural**, v. 40, p. 2107-2114, 2010a.

CUNHA, M. A. et al., Essential oil of *Lippia alba*: a new anesthetic for silver catfish, *Rhamdia quelen*. **Aquaculture**, v. 306, p. 403-406, 2010b.

CUNHA et al. Anesthetic induction and recovery of *Hippocampus reidi* exposed to the essential oil *Lippia alba*. **Neotropical Ichthyology**, v. 9, n. 3, p. 683-688, 2011.

CYMBOROWSKI, B. Introduction to circadian rhythms. IN: KULCZYKOWSKA, E; POPEK, W.; KAPOOR, B. G. (Editors) **Biological clock in fish**. Enfield, NH: Science Publishers, 2010, p. 1-7, cap. 1.

DE TOLLA, L. J. et al. Guidelines for the care and use of Fish in research. **ILAR Journal**, v. 37, n. 4, p. 159 – 173, 1995.

DEL POZO, A. et al. Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal Europa seabass. **Physiology & Behavior**, v. 106, n. 4, p. 446-450, 2012.

DIBNER, C.; SCHIBLER, U.; ALBRECHT, U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. **Annual Review of Physiology**, v. 72, p. 517-549, 2010.

ELLIS, T. et al. Cortisol and finfish welfare. **Fish Physiology and Biochemistry**, v. 38, n. 1, p. 163-188, 2011.

EL-HAWARY, S. S. et al. Bioactivities, phenolic compounds and in-vitro propagation of *Lippia citriodora* Kunth cultivated in Egypt. **Bulletin of Faculty of Pharmacy, Cairo University**, p. 1110-1016, 2012.

EUGENOL. Maquira, 2014. Bula de remédio.

FAÇANHA, M. F.; GOMES, L. C. A eficácia do mentol como anestésico para tambaqui (*Colossoma macropomum*, Characiformes: Characidae). **Acta Amazonica**, v. 35, p. 71-75, 2005.

FELICIANO, A. et al. Feeding time synchronizes clock genes rhythmic expression in brain in liver of goldfish (*Carassius auratus*). **Journal of Biological Rhythms**, v. 26, v. 1, p. 24-33, 2011.

FOGLIARINI, C. O. et al. Anesthesia of *Epinephelus marginatus* with essential oil of *Aloysia polystachya*: an a pproach on blood parameters. **Anais da Academia Brasileira de Ciências**, v. 89, n. 1, p. 445-456, 2017.

GERWICK, L.; DEMERS, N. E.; BAYNE, C. J. Modulation of stress hormones in rainbow trout by means of anesthesia, sensory deprivation and receptor blockade. **Comparative Biochemistry and Physiology Part A**, v. 124, p. 329-334, 1999.

GOMES, L. C. et al. Biologia do jundiá *Rhamdia quelen* (Pisces, Pimelodidae). Uma revisão. **Ciência Rural**, Santa Maria, v. 30, n. 1, p. 179-185, 2000.

GOMES, D. P. et al. Water parameters affect anaesthesia induced by eugenol in silver catfish, *Rhamdia quelen*. **Aquaculture Research**, v. 42, p. 878-886, 2011.

GONÇALVES, A. F. N. et al. Mentol e eugenol como substitutos da benzocaína na indução anestésica de juvenis de pacu. **Acta Scientiarum (Animal Sciences)**, v. 30, p. 339-344, 2008.

GRESSLER et al., Silver catfish *Rhamdia quelen* immersion anaesthesia with essential oil of *Aloysia triphylla* (L'Hérit) Britton or tricaine methanesulfonate: effect on stress response and antioxidant status. **Aquaculture Research**, v. 45, n. 6, p. 1061-1072, 2014.

HAJEK, G. T. The anaesthetic-like effect of tea tree oil in common carp *Cyprinus carpio* L. **Aquaculture Research**, v. 42, p. 296-300, 2011.

HELDWEIN, et al. S-(+)-Linalool from *Lippia alba*: sedative and anesthetic for silver catfish (*Rhamdia quelen*). **Veterinary Anaesthesia and Analgesia**, v. 41, n. 1, p. 621-629, 2014.

HENNEBELLE, T. et al. Review: Ehtnopharmacology of *Lippia alba*. **Journal of Ethnopharmacology**, v. 116, p. 211-222, 2008.

INOUE, L. A. K. A. et al. Effects of clove oil on the stress response of matrinxã (*Brycon cephalus*) subjected to transport. **Acta Amazonica**, v. 35, n. 2, 2005.

INOUE, L. A. K. A. et al. Avaliação de respostas metabólicas do tambaqui exposto ao eugenol em banhos anestésicos. **Acta Amazonica**, v. 41, p. 327-332, 2011.

JÉGU, M.; INGENITO, L. F. S. 2007. Serrasalminae. Pp. 40-43. In: Buckup, P. A., N. A. Menezes E M. S. Ghazzi (Eds.). **Catálogo das espécies de peixes de água doce do Brasil**. Rio de Janeiro, Museu Nacional, 195p.

JULIÃO, L. S. et al. Cromatografia em camada fina de extratos de três quimiotipos de *Lippia alba* (Mill) N. E. Br. (erva-cidreira). **Revista de Brasileira de Farmacognosia**, v. 13, p. 36-38, 2003.

KREYDIYYEH, S. I. et al. Aniseed oil increases glucose absorption and reduces urine output in the rat. **Life Science**, v. 74, p. 663-673, 2003.

LÓPEZ-OLMEDA, J. F; SÁNCHEZ-VÁZQUEZ, F. J. Feeding rhythms in fish: from behavioural to molecular approach. In: KULCZYKOWSKA, E.; POPEK, W.; KAPOOR, B. G. (Editors). **Biological clock in fish**. Enfield, NH: Science Publishers, 2010, p. 155-184, cap. 8.

LORENZI, H.; MATOS, F. J. A. **Plantas medicinais do Brasil: nativas e exóticas**. Ed. Nova Odessa: Plantarum. 2008. 554 p.

MANZANILLA, E. G. et al. Effect of plant extracts and formic acid n the intestinal equilibrium of early-weaned pigs. **Journal Animal Science**, v. 82, p. 3210-3218, 2004.

MARICCHIOLO, G.; GENOVESE, L. Some contributions to knowledge of stress response in innovative species with particular focus on the use of the anaesthetics. **The Open Marine Biology Journal**, v. 5, p. 24 – 33, 2011.

MARQUES, N.; MENNA-BARRETO, L. **Cronobiologia: princípios e aplicações**. 3 ed. São Paulo, EDUSP/Editora Fiocruz, 2003. 435p.

MAYWOOD, E. S. et al. Genetic and molecular analysis of the central and peripheral circadian clockwork of mice. **Cold Spring Harbor symposia on quantitative biology**, v. 72, p. 85-94, 2007.

MAZEAUD, M. M.; MAZEAUD, F.; DONALDSON, E. M. Primary and secondary effects of stress in fish: some new data with a general review. **Transactions of the American Fisheries Society**, v. 106, p. 201 – 212, 1977.

MAZANDARANI, M.; HOSEINI, S. M. Menthol and 1,8-cineole as new anaesthetics in common carp, *Cyprinus carpio* (Linnaeus, 1758). **Aquaculture Research**, v. 48, n. 6, p. 3041-3051, 2017.

MAZANDARANI, M.; HOSEINI, S. M. Anesthesia of juvenile Persian sturgeon, *Acipenser persicus*, Borodin 1897, by peppermint, *Mentha piperita*, extract – Anesthetic efficacy, stress response and behavior. **International Journal of Aquactic Biology**, v. 5, n. 6, p. 393-400, 2018.

MIRGHAED, A. T. et al. Rainbow trout (*Oncorhynchus mykiss*) anesthesia with myrcene: efficacy and physiological responses in comparison with eugenol. **Fish Physiology and Biochemistry**, v. 44, n. 3, p. 919-926, 2018.

MORAES, M. F. P. G. de, BARBOLA, I. de F.; DUBOC, L. F. Feeding habits and morphometry of digestive tracts of *Geophagus brasiliensis* (Osteichthyes, Cichlidae), in a lagoon of high Tibagi River, Paraná State, Brazil, **Publicatio UEPG: Ciências Biológicas e da Saúde**, v. 10, p. 37-45, 2004.

MOREIRA, A. G. L. et al. Eficácia do eugenol extraído da planta *Eugenia aromatica* como anestésico para realização de biometrias em adultos de tilápia do Nilo (*Oreochromis niloticus*). **Acta Scientiarum (Animal Sciences)**, v. 32, p. 419-423, 2010.

MORGAN, E. Ecological significance of biological clocks. **Biological Rhythms Research**, v. 35, n. 1-2, p. 3-12, 2004.

MUHL, A.; LIEBERT, F. Growth and parameters of microflora in intestinal and faecal samples of piglets due to application of phytogenic feed additive. **Journal of Animal Physiology and Animal Nutrition**, v. 91, p. 411-418, 2007.

OLIVEIRA, J. R. et al. Cloreto de sódio, benzocaína e óleo de cravo-da-índia na água de transporte de tilapia-do-nilo. **Revista Brasileira de Zootecnia**, v. 38, n. 7, p. 1163-1169, 2009.c

PARODI, T. V. et al. The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). **Comparative Biochemistry and Physiology parte C: Toxicology E Pharmacology**, v. 155, p. 462-468, 2012.

PARODI, T. V. et al. Anesthetic activity of the essential oil of *Aloysia triphylla* and effectiveness in reducing stress during transport of albino and gray strains of silver catfish, *Rhamdia quelen*. **Fish Physiology Biochemistry**, v. 40, p. 323-334, 2014.

PASCUAL, M. E. et al. *Lippia*: traditional uses, chemistry and pharmacology: a review. **Journal of Ethnopharmacology**, v. 76, p. 201 – 214, 2001.

PAULUS, D. et al. Teor e composição química do óleo essencial e crescimento vegetativo de *Aloysia triphylla* em diferentes espaçamentos e épocas de colheita. **Revista Ceres**, v. 60, p. 372-379, 2013.

PEREIRA, D. S.; TUFIK, S.; PEDRAZZOLI, M. Moléculas que marcam o tempo: implicações para fenótipos circadianos. **Revista Brasileira de Psiquiatria**, v. 31, n. 1, p. 63-71, 2009.

PLATEL, K.; SRINIVASAN, K. Digestive stimulant action of spices: A myth or reality? **Indian Journal Medical Research**, v. 119, p. 167-179, 2004.

PORTZ, D. E.; WOODLEY, C. M.; CECH JR, J. J. Stress-associated impacts of short-term holding on fishes. **Reviews in Fish Biology and Fisheries**, v. 16, n. 2, p. 125-170, 2006.

POUILLY, M. et al. Dietary-morphological relationships in a fish assemblage of the Bolivian Amazonian floodplain. **Journal of Fish Biology**, v. 62, n. 5, p. 1137-1158, 2003.

RAO, R.; PLATEL, K; SRINIVASAN, K. In vitro incluence of spices and spices-active principles on digestive enzymes of rat pancreas and small intestine. **Nahrung**, v. 47, p. 408-412, 2003.

RAUT, J. S.; KARUPPAYIL, S. M. A status review on the medicinal properties of essential oils. **Industrial Crops and Products**, v. 62, p. 250-264, 2014.

REID, S. G.; BERNIER, N. J.; PERRY, S. F. The adrenergic stress response in fish: control of catecholamine storage and release. **Comparative Biochemistry and Physiology Part C**, v. 120, p. 1-27, 1998.

ROSBASH, M. Molecular control of circadian rhythms. **Current opinion in genetics E development**, v. 5, n. 5, p. 662-668, 1995.

ROSS, G. L.; ROSS, B. R. Stress in aquatic animals. In: Ross, G. L. (Ed.), **Anaesthetic and sedative techniques for aquatic animals**, 2nd. ed., vol. 77, Blackwell Science LTD., Bod min, Cornwall, 2008, p. 5 – 21.

ROUBACH, R. et al. Eugenol as an efficacious anaesthetic for tambaqui, *Colossoma macropomum* (Cuvier). **Aquaculture Research**, v. 36, p. 1056-1061, 2005.

SABINO, J.; CASTRO, R. M. C. Alimentação, período de atividade e distribuição espacial dos peixes de um riacho da floresta Atlântica (Sudeste do Brasil). **Revista Brasileira de Biologia**, v. 50, p. 23-36, 1990.

SÁNCHEZ, J. A.; SÁNCHEZ-VÁZQUEZ, F. J. Feeding entrainment of daily rhythms of locomotor activity and clock gene expression in zebrafish brain. **Cronobiology International**, v. 26, n. 6, p. 1120-1135, 2009.

SÁNCHEZ-VÁZQUEZ F. J.; ZAMORA, S.; MADRID, J. A. Light-dark and food restriction cycles in sea bass: effect of conflicting zeitgebers on demand-feeding rhythms. **Physiology E Behaviour**, v. 58, n. 4, p. 705-714, 1995.

SANTURIO, J. M. et al. Atividade antimicrobiana dos óleos essenciais de orégano, tomilho e canela frente a sorovares de *Salmonella enterica* de origem avícola. **Ciência Rural**, v. 37, p. 803-808, 2007.

SANTOS, G.; FERREIRA, E.; ZUANON, J. **Peixes comerciais de Manaus**. Ibama, Manaus, Brasil, 2006, 144 p.

SCHRECK, C. B.; TORT, L. The Concept of Stress in Fish. IN: SCHRECK, C. B.; TORT, L.; FARREL, A. P.; BRAUNER, C. J. (Editors). **Biology of stress in fish**. United Kingdom: Academic Press, 2006, p. 1-35.

SCHILLING, T. F. **The morphological of larval and adult zebrafish in Nüsslein-Volhard E Dahm**. Zebrafish – A practical approach. Oxford: UK, 2002.

SILVA JUNIOR, A. A. **Plantas medicinais**. Itajaí: EPAGRI, 1998.

SILVA, S. S. et al. Anesthetic activity of Brazilian native plants in silver catfish (*Rhamdia quelen*). **Neotropical Ichthyology**, v. 11, n. 2, p. 443-451, 2013.

SILVEIRA, U. S.; LOGATO, P. V. R.; PONTES, E. C. Fatores estressantes em peixes. **Revista Eletrônica Nutritime**, v. 6, p. 1001-1017, 2009.

SIMÕES, L. N.; GOMES, L. C. Eficácia do mentol como anestésico para juvenis de tilápia-do-nilo (*Oreochromis niloticus*). **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 61, p. 613-620, 2009.

SMALL, B. C. Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*. **Aquaculture**, v. 218, p. 177 – 185, 2003.

SNEDDON, L. U. Clinical anesthesia and analgesia in fish. **Journal of Exotic Pet Medicine**, v. 21, p. 32-43, 2012.

SPENCE, R. et al. The behavior and ecology of the zebrafish, *Danio rerio*. **Biological Reviews**, v. 83, p. 13 – 34, 2008.

SPEZIALI, M. G. From aromas and perfumes, the market of the smell industry. **Química Nova**, v. 35, p. 861-864, 2012.

SUMMERFELT, R. C.; SMITH, L. S. Anesthesia, surgery and related techniques. In: SCHERK, C.B., MOYLE, P. B. Methods for fish biology, **American Fishery Society**, 684 p., 2000.

TEIXEIRA, R. R. et al. Essential oil of *Aloysia triphylla* in Nile tilapia: anaesthesia, stress parameters and sensory evaluation of fillets. **Aquaculture Research**, v. 48, n. 7, 2017.

TONDOLO, J. S. M. et al. Anesthesia and transport of fat snook *Centropomus parallelus* with the essential oil of *Nectandra megapotamica* (Spreng.) Mez. **Neotropical Ichthyology**, v.11, p. 667-674, 2013.

TONI, C. et al. Fish anesthesia: effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). **Fish Physiology and Biochemistry**, v. 40, n. 3, p. 701-714, 2014.

TONI, C. et al. Stress response in silver catfish (*Rhamdia quelen*) exposed to the essential oil of *Hesperozygis ringens*. **Fish Physiology and Biochemistry**, v. 41, n. 1, p. 129-138, 2015a.

TONI, C. et al. Sedative effect of 2-phenoxyethanol and essential oil of *Lippia alba* on stress response in gilthead sea bream (*Sparus aurata*). **Research in Veterinary Science**, v. 103, p. 20-27, 2015b.

TORT, L. Stress and immune modulation in fish. **Developmental and Comparative Immunology**, v. 35, p. 1366 – 1375, 2011.

URBINATI, E. C. et al. Loading and transport stress in juvenile matrinxã (*Brycon cephalus*) at various densities. **Aquaculture**, v. 229, n. 1-4, p. 389-400, 2004.

VATINE, G. et al. It's time to swin! Zebrafish and the circadian clock. **FEBSLetters**, v. 585, n. 10, p. 1485-1494, 2011.

VELARDE, E. et al. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of *Period* and *Cryptochrome* transcripts in retina, liver and gut. **Journal of Biological Rhythms**, v. 24, n. 2, p. 104-113, 2009.

VERA, L. M. et al. Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. **Physiology E Behavior**, v. 90, n. 2-3, p. 518-524, 2007.

VERA, L. M. et al. Circadian rhythms of locomotor activity in the Nile Tilapia (*Oreochromis niloticus*). **Chronobiology International**, v. 26, n. 4, p. 666-681, 2009.

VERA, L. M. et al. Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). **Cronobiology International**, v. 30, n. 5, p. 649-661, 2013.

WEBER, E. S. Fish analgesia: pain, stress, fear aversion, or nociception? **Veterinary Clinics of North America: Exotic Animal Practice**, v. 14, p. 21 – 32, 2011a.

WEBER, R. A. et al. Effects of acute exposure to 2-phenoxyethanol, clove oil, MS-222 and metomidate on primary and secondary stress response in Senegalese sole (*Solea senegalensis* Kaup 1858). **Aquaculture**, v. 321, p. 108-112, 2011b.

ZAHL, I. H.; SAMUELSEN, O.; KIESSLING, A. Anaesthesia of farmed fish: Implications for welfare. **Fish Physiology and Biochemistry**, v. 38, p. 201-218, 2011.

ZEPPENFELD, C. C. et al. Physiological and biochemical responses of silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton. **Aquaculture**, v. 418-419, p. 101-107, 2014.

ZHDANOVA, I. V.; REEBS, S. G. Circadian rhythms in fish. IN: SLOMAN, K. A.; WILSON, R. W.; BALSHINE, S. (Editors). **Fish Physiology: Behaviour and Physiology of Fish**. 1 ed. EUA: Academic Press, 2006, p. 197-239, cap. 6.