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

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MORFOFISIOLOGIA DE JUNDIÁS SEDADOS OU ANESTESIADOS COM MS222, ÓLEO ESSENCIAL DE *Aloysia triphylla* E PROPOFOL

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Farmacologia.**

Orientador: Prof. Dr. Bernardo Baldisserotto

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Aprovada em 24 de junho de 2016:

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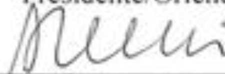

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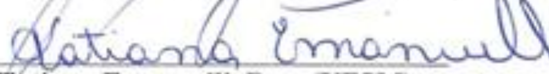

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2016

RESUMO

MORFOFISIOLOGIA DE JUNDIÁS SEDADOS OU ANESTESIADOS COM MS222, ÓLEO ESSENCIAL DE *Aloysia triphylla* E PROPOFOL

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Um dos pilares para a otimização do manejo de peixes cativos é a minimização do estresse, sendo os sedativos e anestésicos ferramentas habitualmente empregadas para obtenção deste objetivo. Entretanto, estes fármacos podem causar alterações que eventualmente se tornam deletérias ao animal se ultrapassarem o limite fisiológico. Neste contexto, o presente trabalho investigou o uso de uma substância de origem natural, o óleo essencial (OE) de *Aloysia triphylla*, e de dois anestésicos sintéticos, o metanosulfonato de tricaína (MS222) e o propofol, em jundiás (*Rhamdia quelen*). Os testes incluíram anestesia (OE e MS222), para uso em manipulações intensas ou invasivas, e sedação (propofol), para uso em procedimentos como o transporte. Análises dos efeitos do MS222, o protótipo de anestésico para peixes, e do OE de *A. triphylla* demonstraram que anestesia com a substância natural apresentou maiores benefícios por evitar a lipoperoxidação e limitar a secreção de cortisol e a perda de íons, apesar de os tempos de indução e recuperação terem sido mais curtos com o anestésico tradicional. O propofol como sedativo por até 12 horas não aumentou a liberação de cortisol e as alterações observadas em índices hematológicos, bioquímicos e hidrominerais foram limitadas. Ainda, a avaliação da morfologia branquial indicou que ionorregulação e ventilação não foram afetadas pelo propofol, enquanto que marcadores de estresse oxidativo evidenciaram a ausência de dano peroxidativo em órgãos vitais, encéfalo e fígado, devido à indução de alguns componentes do sistema de defesa antioxidante, especialmente da enzima superóxido dismutase, na presença do fármaco. Estes resultados indicam que o OE de *A. triphylla* e o propofol representam alternativas promissoras para uso na piscicultura brasileira, uma vez que anestésicos rotineiramente administrados a peixes em outros países, como o MS222, não estão disponíveis para compra no Brasil. Além disso, o desempenho do MS222 foi inferior àquele do OE, o que demonstra que tempos curtos de indução e recuperação não necessariamente significam menos prejuízo fisiológico. Estudos como os descritos neste trabalho contribuem para a preservação do bem-estar do animal mantido em cativeiro por sugerirem ações possíveis para redução do estresse.

Palavras-chave: *Rhamdia quelen*. Piscicultura. Transporte. Estresse.

ABSTRACT

MORPHOPHYSIOLOGY OF SIVER CATFISH SEDATED OR ANTESTHETIZED WITH MS222, ESSENCIAL OIL OF *Aloysia triphylla* AND PROPOFOL

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One of the pillars for optimizing cultured fish handling is stress reduction, and sedatives and anesthetics are tools usually employed in order to reach such goal. However, these drugs may cause physiological changes that can be deleterious to the animal if surpassing a physiological threshold. In this context, the current work investigated the use of a substance of natural origin, the essential oil (EO) of *Aloysia triphylla*, as well as of two synthetic drugs, tricaine methanesulfonate (MS222) and propofol, in silver catfish (*Rhamdia quelen*). The trials involved anesthesia (EO and MS222), for application in intensive or invasive manipulations, and sedation (propofol), to be used in procedures such as fish transport. Analyses of the effects of MS222, the prototypical fish anesthetic, and of the EO of *A. triphylla* showed that anesthesia with the naturally occurring substance presented more benefits for it prevented lipoperoxidation and limited cortisol secretion and ionic loss, although induction and recovery times were shorter with the traditional drug. Propofol as sedative for up to 12 hours did not increase cortisol secretion and alterations observed in hematological, biochemical and hydromineral indexes were limited. Moreover, assessment of gill morphology indicated that ionregulation and ventilation were not affected by propofol, while oxidative stress markers evidenced absence of peroxidative damage in vital organs, brain and liver, due to induction of some components of the antioxidant system, specially the enzyme superoxide dismutase, in the presence of the drug. These findings indicate that the EO of *A. triphylla* and propofol represent promising alternatives to be used in Brazilian fish culture, since anesthetics routinely applied in fish in other countries, such as MS222, are not commercially available in Brazil. In addition, the performance of MS222 was inferior when compared to that of the EO, which demonstrates that short induction and recovery periods do not necessarily mean less physiological burden. Studies as the ones described in this work contribute to welfare preservation of captive animal since possible actions to reduce stress are suggested.

Keywords: *Rhamdia quelen*. Fish culture. Transport. Stress.

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1 INTRODUÇÃO

1.1 AQUICULTURA

A aquicultura mundial é responsável pela produção de quase 50% do pescado utilizado para consumo humano. O Brasil vem colaborando ativamente para a expansão da cadeia do peixe cultivado, principalmente no que se refere ao uso de águas continentais, sendo o 2º maior produtor na América Latina, atrás do Chile. O primeiro Censo Aquícola do Brasil, desenvolvido pelo Ministério da Pesca e Aquicultura (MPA, 2013) e referente ao ano de 2008, identificou que 15 espécies de peixes são cultivadas na aquicultura marinha e 62 espécies são criadas em água doce, sendo a tilápia nilótica *Oreochromis niloticus* a principal espécie atualmente cultivada no país. De acordo com o Boletim Estatístico da Pesca e Aquicultura (MPA, 2011), 45% do pescado brasileiro originou de águas cultivadas em 2011. Em 2012, o país se destacou como o 12º maior produtor mundial de pescado, incluindo peixes, crustáceos e moluscos, totalizando 707.661 toneladas. Este valor corresponde a 1,1 % da produção da aquicultura global, a qual é liderada pela China com uma produção equivalente a 41.108.306 toneladas (61,7%). A aquicultura mundial produziu 66.633.253 toneladas em 2012 (FAO, 2014).

Segundo o MPA, o Brasil tem potencial para se tornar um país líder na produção de pescado por apresentar uma extensa costa marítima, bilhões de metros cúbicos de água doce, distribuídos especialmente nas doze grandes bacias hidrográficas, terras disponíveis e condições ambientais e climáticas favoráveis. As regiões Nordeste e Sul se destacam como as maiores produtoras de pescado, totalizando 727.720 toneladas em 2009, e a região Norte é a grande promessa do setor, devido à diversidade de espécies, abundância de rios e o elevado consumo per capita deste tipo de alimento. O MPA regulamenta e apoia o desenvolvimento responsável e sustentável da pesca de captura e da aquicultura no Brasil seja ela amadora, artesanal ou industrial. O órgão almeja um incremento de 20% na produção brasileira de pescado, alcançando 3 milhões de toneladas até 2020, sendo 2 milhões de toneladas oriundas da aquicultura (MPA, 2015).

O pescado é o alimento mais amplamente comercializado, especialmente em países em desenvolvimento, onde pode representar até 50% do total de mercadorias negociadas. Na década de 60, o consumo mundial de carne de peixe era de 9,9 quilos por indivíduo; já em 2012, este número havia subido para 19,2 quilos. Este aumento ocorreu em grande parte pela maior diversidade de peixes disponível para o consumidor devido à expansão nas

importações. No Brasil, no entanto, o consumo per capita em 2015 foi de 10,6 quilos, abaixo do mínimo recomendado pela Organização Mundial da Saúde (OMS), 12 quilos/ano. A carne de peixe é a proteína animal mais saudável e nutritiva, sendo um importante item na dieta humana por promover a saúde e reduzir a fome. Além disso, a pesca auxilia na diminuição da pobreza, criando empregos a uma taxa mais alta do que o crescimento da população. Estima-se que esta atividade seja responsável pelo sustento de aproximadamente 12% da população mundial (FAO, 2014; OMS, 2015).

Sendo responsável por aproximadamente 50% da produção aquícola mundial, a piscicultura requer constante pesquisa e inovação. Com todo este potencial, o aprimoramento tecnológico, englobando desde as etapas iniciais de cultivo até a entrega da carne ao consumidor final, é de fundamental importância para assegurar a qualidade do produto e a lucratividade (FAO, 2014). Uma vez que os peixes são facilmente estressáveis, a redução no ganho de peso, no crescimento e na resistência a patógenos, ou até mesmo o óbito, que podem decorrer de manejo inadequado, representam alguns dos principais entraves da atividade (BARCELLOS, SOUZA, WOEHL, 2000). No entanto, o uso de técnicas apropriadas durante os procedimentos inerentes à cadeia produtiva da piscicultura pode auxiliar a preservar o bem-estar do animal e minimizar a queda na produção (SMALL, 2004; MARICCHIOLO, GENOVESE, 2011).

1.1.1 O jundiá

O jundiá *Rhamdia quelen* (Quoy & Gaimard 1824) é um teleósteo de água doce que pertence à ordem Siluriformes e à família Heptapiteridae. É um peixe neotropical, ocorrendo do sudeste do México ao centro da Argentina, onde se abriga em tocas em águas mais calmas e sai à noite à procura de alimento (SILFVERGRIP, 1996; GOMES et al., 2000; BALDISSEROTTO, 2004). Apesar de ser onívoro, o jundiá apresenta tendência piscívora, mas também se alimenta de crustáceos, insetos, restos vegetais e detritos orgânicos (GUEDES, 1980; MEURER, ZANIBONI FILHO, 1997). É um peixe de couro, variando da coloração marrom avermelhado claro a cinza ardósia. A variedade albina também pode ser encontrada, sendo esta comercializada como peixe ornamental (GOMES et al., 2000; BALDISSEROTTO, 2009).

É uma das espécies nativas mais estudadas no Brasil (GOMES et al., 2000; COLDEBELLA, RADÜNZ NETO, 2002; BALDISSEROTTO, 2004; MIRON et al., 2008; CUNHA et al., 2010a,b; GARCIA et al., 2011a,b; BECKER et al., 2012; BARCELLOS et al.,

2012; KOAKOSKI et al., 2012; EMANUELLI, PICCOLO, 2013; SACCOL et al., 2013; SUTILI et al., 2013, 2015; PARODI et al., 2014; RIFFEL et al., 2014; SALBEGO et al., 2014; TONI et al., 2014, 2015; COSTA, et al., 2015; SOUZA et al., 2015; GOLOMBIESKI et al., 2016; PES et al., 2016). Isso reflete na sua crescente importância econômica no sul do país: os resultados elucidativos e promissores das pesquisas, juntamente com as vantagens zootécnicas do jundiá, tornaram sua produção uma atividade em franca expansão. Os trabalhos relacionados à sua biologia e fisiologia, parâmetros da água para seu melhor desempenho, dieta, parasitismo, transporte, anestesia e qualidade da carne, entre outros, formam um pacote tecnológico para a otimização do seu cultivo, acrescentando maior rentabilidade à produção.

A anestesia destaca-se como um dos temas mais estudados em jundiá na atualidade, especialmente pelo Laboratório de Fisiologia de Peixes (UFSM). Em geral avalia-se a possibilidade de se utilizar determinadas substâncias de origem natural e sintética para a espécie a partir de dados obtidos durante os testes, como os tempos de indução e recuperação da anestesia, as concentrações efetivas e parâmetros fisiológicos visíveis, como batimento opercular. Além disso, são coletadas amostras biológicas, como sangue e tecidos, a fim de avaliar a toxicidade sistêmica destes fármacos em análises hematológicas, bioquímicas, histológicas e referentes a marcadores de estresse oxidativo, entre outros. O intuito destes estudos é indicar substâncias que possam ser utilizadas para anestesiar a espécie em procedimentos como biometria, coleta de biópsias e transporte, por exemplo, visando minimizar o estresse, aumentar a precisão das técnicas, reduzir os riscos de dano ou óbito e conferir valores éticos ao trato com estes animais (CUNHA et al., 2010a,b; GOMES et al., 2011; BECKER et al., 2012; BENOVIT et al., 2012, 2015; GRESSLER et al., 2012; SILVA et al., 2012, 2013a,b; HELDWEIN et al., 2014; PARODI et al., 2014; SALBEGO et al., 2014; TONI et al., 2014, 2015; GARLET et al., 2016).

O jundiá é um peixe de bom crescimento e que se reproduz com facilidade. Por ser euritérmico, tolera variações de temperatura de 15 a 35° C (BALDISSEROTTO, 2004; ZANIBONI FILHO, 2004). Devido à sua característica rusticidade, apresenta boa adaptação ao alimento artificial extrusado ou flutuante em cativeiro. Estas e outras características permitem que seja criado em policultivo com tilápia ou carpa *Cyprinus carpio* (SILVA et al., 2006) ou em monocultivo intensivo em tanques redes (BARCELLOS et al., 2004a) ou escavados (FRACALOSSI et al., 2004). Seu filé sem espinhos tem alto rendimento e boa qualidade e sabor, sendo bem aceito pelo mercado consumidor, inclusive para a pesca esportiva (CARNEIRO, 2004; AMARAL JUNIOR et al., 2013).

O Censo Aquícola Nacional de 2008 constatou a existência de 537 criatórios comerciais de jundiá no Rio Grande do Sul, 481 em Santa Catarina, 160 no Paraná e 9 em São Paulo. O cultivo da espécie no Rio Grande do Sul e em Santa Catarina é feito predominantemente em açudes, enquanto no Paraná e em São Paulo os viveiros são mais utilizados. Nestas estruturas de produção, o monocultivo para criação de formas jovens é geralmente o sistema adotado em São Paulo. Já na região Sul, destaca-se o policultivo visando à engorda. Em 77% das unidades produtivas nacionais, o cultivo tem como finalidade a produção de animais, sendo o restante direcionado para pesque-pague. O comércio de jundiá vivo predomina no país (43%), seguido pelo produto fresco (36%), eviscerado (8%), congelado (2%) e filé (1%). Segundo o Boletim Estatístico da Pesca e Aquicultura (MPA, 2011), a produção de jundiás oriunda da aquicultura continental foi de 1.747,3 toneladas em 2011, o que correspondeu a 0,28% da produção nacional de peixes em cativeiro (628.704,3 toneladas). Já a pesca extrativa da espécie, que segundo o boletim Estatística da Pesca Brasil 2007 (IBAMA) ocorre em Rondônia, Acre, Roraima e Sergipe, Santa Catarina e Rio Grande do Sul, atingiu 354,7 toneladas no mesmo ano (MPA, 2011).

O 1º Anuário Brasileiro da Pesca e Aquicultura (ACEB, 2014) destaca o jundiá como uma das principais espécies nativas com potencial para utilização na piscicultura, juntamente com dourado *Salminus brasilensis*, jaú *Paulicea luetkeni*, matrinxã *Brycon amazonicus*, piau *Leporinus spp*, pintado *Pseudoplatystoma corruscans* e pirarucu *Arapaima gigas*. Mas a participação destes peixes nativos na piscicultura brasileira ainda é pequena, sendo de aproximadamente 20%.

Apesar da expansão da criação de jundiá em cativeiro, há alguns entraves a serem solucionados, como a alta mortalidade em decorrência de parasitos ciliados e infecções bacterianas (GARCIA et al., 2011a,b; SUTILI et al., 2013, 2015). Suas exigências nutricionais também devem ser mais amplamente compreendidas a fim de aprimorar seu desempenho (RADÜNZ NETO, BORBA, 2013). A Rede Jundiá, que envolve instituições como EPAGRI, UFSC, UFSM, UPF, ESALQ, IPESCA e UFPR, foi criada em 2011 justamente com o intuito de gerar informação acerca da espécie e elucidar questões pertinentes a sua cadeia produtiva, com o apoio do CNPq e da FAPESC.

1.1.2 Principais tipos de manejo de peixes

A criação de peixes envolve uma extensa coleção de manejos, os quais devem ser realizados com base em princípios importantes como a manutenção da saúde animal, a

sustentabilidade ambiental dos sistemas aquícolas e a rentabilidade da atividade. Boas práticas de manejo como a redução da densidade de estocagem, o uso de rações de boa qualidade, a manutenção da qualidade da água e os cuidados com o solo têm sido difundidas a fim de preservar o bem-estar animal, gerar menos impacto ambiental e produzir maiores vantagens para os produtores (EMBRAPA, 2003).

Dentre os vários procedimentos realizados com os animais, a reprodução induzida destaca-se por envolver intensa manipulação dos reprodutores, o que causa estresse. Inicialmente, os peixes são capturados e retirados da água para aplicação hormonal. Algumas horas após a inoculação do indutor é feita uma nova captura e exposição ao ar para a extrusão de gametas (AMARAL JUNIOR et al., 2013). Este manuseio excessivo pode levar a óbito se não for realizado com a devida cautela. Da mesma forma, investigações científicas e a prática da medicina ictícola também requerem manipulação demasiada. A coleta de amostras biológicas e de biópsias, a biometria e a realização de cirurgias são momentos estressantes para os peixes e que podem gerar dor. A presença de nociceptores em peixes, além de outras evidências de que são capazes de sentir dor, os torna vulneráveis quando procedimentos invasivos são realizados (SNEDDON, 2015). Por isso, qualquer uma destas intervenções deve ser realizada seguindo protocolos anteriormente estabelecidos e baseados em conhecimento prévio dos executores, utilizando-se técnicas que otimizem o tempo, preservem a sanidade animal e assegurem o sucesso da operação (GILDERHUS, MARKING, 1987; COYLE, DURBOROW, TIDWELL, 2004; NEIFFER, STAMPER, 2009).

Outro procedimento estressante no sistema de produção é a despesca, pois causa exaustão demasiada nos animais. As repetidas passagens de rede nos tanques, a suspensão de argila e material orgânico na água e o confinamento na rede por períodos prolongados provocam lesões no corpo e nas brânquias e desencadeam reações de estresse, possivelmente levando a um desequilíbrio osmorregulatório e à depressão do sistema imunológico. Como resultado, o desempenho zootécnico fica comprometido e a mortalidade durante e após o transporte realizado subsequentemente à despesca, bem como durante o confinamento para depuração pré-transporte, torna-se elevada (BAKER, GOBUSH, VYNNE, 2013).

O transporte, que se destaca por ser uma das mais importantes etapas na piscicultura, deve ser bem executado a fim de premiar as boas práticas anteriormente empregadas no processo de produção. Se realizado de maneira incorreta, pode resultar em mortalidade dos animais tanto durante o procedimento quanto após a transferência para as novas instalações (CARMICHAEL, TOMASSO, SCHEDLER, 2001; TREASURER, 2010; BUIN et al., 2013). Isto ocorre se for feito em dias com temperatura extrema, sem observar a densidade de

estocagem apropriada, sem realização de depuração prévia e em água de má qualidade, por exemplo (BARCELLOS, SOUZA, WHOEL, 2000). A manutenção dos parâmetros de qualidade da água em níveis adequados é fator primordial. Oxigênio, temperatura e pH estáveis e na faixa de preferência da espécie podem minimizar os efeitos deletérios causados por substâncias potencialmente tóxicas, tais como amônia não-ionizada (NH_3) e dióxido de carbono (CO_2), as quais tendem a aumentar durante o transporte (AMEND et al., 1982; YANONG, 2006). O transporte de peixes com alta infestação de parasitos também colabora para o aumento das perdas (CARNEIRO, URBINATI, MARTINS, 2002).

Os peixes podem ser transportados em sistema aberto ou fechado. No sistema aberto os animais são mantidos em caixas ou tanques apropriados, com fornecimento permanente de oxigênio ou de ar. Os recipientes mais utilizados possuem de 500 a 1000 litros de capacidade e são montados sobre caminhões ou barcos adaptados para o transporte de peixes, com sistemas mecânicos contínuos de aeração e/ou circulação de água. Porém, muitas vezes o transporte é feito em embalagens menores dentro de veículos particulares. Já no sistema fechado, os animais são distribuídos em sacos plásticos específicos para esta finalidade, os quais geralmente tem a capacidade total para 30 litros a fim de facilitar a manipulação. Os sacos são preenchidos com água até 30% da sua capacidade, sendo o restante completado com oxigênio puro. As embalagens são então amarradas ou seladas e acondicionadas de maneira a evitar o extravasamento de água e também a deterioração da mesma em decorrência de fatores externos (BERKA, 1986; GOLOMBIESKI, 2004; BUIN et al., 2013). Ao final do transporte os peixes devem ser aclimatados à água do local de estocagem, sendo a água utilizada para o transporte misturada gradualmente à dos reservatórios do destino. Assim evita-se a mortalidade dos animais em decorrência da mudança brusca de ambiente. Portanto, o transporte de peixes vivos é um dos pontos chaves da produção ao qual o estresse está necessariamente atrelado por reunir muitas variáveis relacionadas ao animal, ao ambiente e aos operadores. Por isso deve envolver cuidados que começam dias antes ao procedimento e só acabam alguns dias após o a realocação dos peixes, quando estes já estão aclimatados ao local de destino (BERKA, 1986; GOLOMBIESKI, 2004; BUIN et al., 2013).

1.2 ESTRESSE EM PEIXES: RESPOSTAS PRIMÁRIA, SECUNDÁRIA E TERCIÁRIA

Fatores estressantes, como os relacionados ao sistema produtivo de peixes descritos acima, desencadeam a resposta ao estresse. O sistema neuroendócrino é o principal mecanismo de controle do estresse, configurando a resposta primária (MARICCHIOLO,

GENOVESE, 2011). As células cromafins e interrenais, localizadas na glândula interrenal, são responsáveis pela produção de catecolaminas (principalmente adrenalina) e corticosteroides (principalmente cortisol), respectivamente (BARTON, 2002; WENDELAAR BONGA, 1997). A liberação de catecolaminas decorre da estimulação das células cromafins por meio da inervação ganglionar simpática, (eixo hipotálamo-sistema simpático-células cromafins, HSC), quando o sistema nervoso central (SNC) reconhece um determinado fator como sendo uma ameaça ao organismo. Já o cortisol é liberado como resultado da ativação do eixo hipotálamo-hipófise-interrenal (HHI): o estímulo estressor induz à liberação do hormônio liberador de corticotrofina (CRH) pelo hipotálamo; o CRH age sobre a hipófise, estimulando a produção e a liberação do hormônio adrenocorticotrófico (ACTH); e o ACTH promove a liberação de cortisol pela glândula interrenal (BARTON, 2002; WENDELAAR BONGA, 1997). Pankhurst (2010) descreveu a resposta primária ao estresse em teleósteos como bifásica, compreendendo inicialmente uma latência curta para que haja o aumento transitório nos níveis circulantes de catecolaminas, seguida de uma latência mais longa para o desencadear da elevação dos níveis de cortisol no plasma, porém de efeito mais duradouro. O tempo de permanência e a severidade do estímulo estressor, além das variáveis inerentes à espécie, possuem grande relevância na intensidade da resposta dos hormônios, determinando a magnitude e o tempo de duração da mesma (PICKERING, 1992; CONTE, 2004; MARICCHIOLO, GENOVESE, 2011).

O nível circulante de cortisol é o indicador mais amplamente acessado a fim de mensurar resposta primária ao estresse decorrente da anestesia em peixes (BARTON, IWAMA, 1991; BARTON, 2002). Estudos conduzidos em diversas espécies de peixe e com diferentes anestésicos apontaram elevação de cortisol após exposição à anestesia (ZAHL et al., 2010; MARICCHIOLO, GENOVESE, 2011). Isto ocorre indiretamente, devido à baixa disponibilidade de oxigênio durante o procedimento anestésico pela ventilação branquial insuficiente, ou via estimulação direta do eixo HHI (BOLASINA, 2006). Em contrapartida, outros relatos mostraram não haver alteração neste parâmetro depois de aplicada a anestesia, demonstrando que o fármaco seria capaz de prevenir a liberação de cortisol (IVERSEN et al., 2003; SINK, STRANGE, SAWYERS, 2007).

O aumento dos níveis dos hormônios do estresse no plasma causa uma série de efeitos, os quais correspondem à resposta secundária. Esta compreende alterações metabólicas (ex.: aumento nos níveis circulantes de glicose e lactato), hematológicas (ex.: elevações no hematócrito e na hemoglobina) e hidrominerais (ex.: alterações nas concentrações plasmáticas de íons), entre outras (BARTON, IWAMA, 1991; WENDELAAR BONGA, 1997; CLAUSS,

DOVE, ARNOLD, 2008; MARICCHIOLO, GENOVESE, 2011; VELISEK et al., 2011). A hiperglicemia é uma das características principais da segunda fase da resposta ao estresse e decorre da ação conjunta das catecolaminas e do cortisol na estimulação da gliconeogênese (SLADKY et al., 2001). A nova glicose supre a demanda energética aumentada, sendo também depositada no fígado como glicogênio para prover futuras ações (BARCELLOS, SOUZA, WOEHL, 2000). Além disso, o aumento nos índices circulantes de glicose, bem como de lactato, estão associados com má qualidade da carne (PICKERING, POTTINGER, 1989).

Com relação à resposta terciária, esta decorre de uma intensa e duradoura alteração nos parâmetros relativos à resposta secundária, o que faz com que apareçam efeitos indesejáveis no metabolismo, crescimento, capacidade reprodutiva, comportamento e resistência a doenças (BARTON, IWAMA, 1991; WENDELAAR BONGA, 1997; SOSO et al., 2008). Ocorrem quedas na produção e na lucratividade, uma vez que os peixes não atingem o tamanho e o peso desejados para comercialização, além das perdas resultantes da disseminação de doenças bacterianas, fúngicas e parasitárias nos meios de produção (BARCELLOS, SOUZA, WOEHL, 2000; DAVIS, GRIFFIN, GRAY, 2003; BARCELLOS et al., 2004b).

A fim de minimizar as consequências deletérias de diversas operações estressantes envolvidas na piscicultura, recomenda-se o uso da anestesia como uma ferramenta importante para manter a homeostasia dos peixes (MOMMSEN, VIJAYAN, MOON, 1999; PIRHONEN, SCHRECK, 2003; SMALL, 2004; YANONG, 2006; PARK et al., 2008; PEREIRA-DA-SILVA et al., 2009; MARICCHIOLO, GENOVESE, 2011). No entanto, quando utilizada sem considerar as variáveis que podem influenciar a sua eficácia, a anestesia *per se* pode desencadear o referido conjunto de respostas ao estresse (ROUBACH, GOMES, VAL, 2001; PIRHONEN, SCHRECK, 2003; BOLASINA, 2006; MARICCHIOLO, GENOVESE, 2011). Nesse sentido, faz-se necessário o conhecimento amplo da técnica e de suas variáveis, das particularidades dos fármacos e da espécie a ser anestesiada, evitando-se, assim, resultados indesejáveis.

1.3 ANESTESIA DE PEIXES

Anestésicos são agentes que primeiramente induzem efeito calmante, denominado sedação, seguido por perda de equilíbrio, de mobilidade, de consciência e, finalmente, de ação reflexa (ROSS, ROSS, 2008). Em peixes, a anestesia pode ser obtida através de métodos

físicos, como a hipotermia e a estimulação elétrica, ou químicos, como a administração de fármacos. No entanto, o uso de alternativas não farmacológicas vem sendo desencorajado devido ao aspecto desumano que lhes é inerente. Assim, quando os termos "anestesia" ou "anestésicos" são utilizados ao longo deste trabalho, referem-se à utilização de fármacos. Além disso, como a sedação é a fase inicial da anestesia, por vezes utilizam-se apenas os termos referidos acima, porém com o intuito de englobar também a sedação.

O anestésico deve apresentar rápida ação depressora sobre o SNC, sem complicações posteriores para o peixe (PARK et al., 2008). Segundo Ross e Ross (2008), ocorre uma depressão generalizada do SNC por uma ação nos axônios através da liberação de neurotransmissores ou por alterações na permeabilidade de membrana (ex.: canais iônicos), ou ainda uma combinação destas ações. Esses autores salientaram que o modo preciso de ação de anestésicos em invertebrados e peixes precisa ser mais amplamente elucidado. No entanto, relataram que, com alguns fármacos, parece haver uma relação inversa entre a concentração necessária para induzir certo estágio anestésico e a posição filogenética dos animais, o que faz com que um peixe necessite uma quantidade maior de anestesia que um mamífero para produzir o efeito desejado. Isso pode ser explicado por um fenômeno relacionado à evolução de mecanismos moleculares, podendo ser consequência da presença aumentada de sítios ativos para determinadas formas moleculares nos vertebrados superiores.

1.3.1 Estágios de anestesia em peixes

De acordo com Schoettger e Julin (1967), são cinco os estágios de indução à anestesia em peixes, evidenciados por mudanças comportamentais visíveis (Tabela 1).

Tabela 1- Estágios de indução à anestesia em peixes

Estágio	Descrição	Características
1	Sedação leve	Perda parcial da reação aos estímulos externos.
2	Sedação profunda	Perda parcial do equilíbrio, nenhuma reação aos estímulos externos.
3a	Perda total do equilíbrio	O peixe vira, mas retem a habilidade da natação.
3b	Perda total do equilíbrio	A habilidade da natação pára, mas responde à pressão no pedúnculo caudal.
4	Anestesia	Perda da atividade reflexa, nenhuma reação aos estímulos externos.
5	Colapso medular	O movimento respiratório cessa (morte).

Fonte: Schoettger e Julin, 1967.

A sedação configura os estágios iniciais da anestesia. Para fins de transporte de peixes, por exemplo, sedação profunda é considerada ideal e suficiente, havendo perda de reatividade a estímulos externos e decréscimo no metabolismo, preservando, no entanto, o equilíbrio (COOKE et al., 2004). Se este for totalmente perdido, o balanço da água durante o transporte pode levar ao aparecimento de lesões devido ao choque dos peixes entre si e contra a lateral dos recipientes, no caso de transporte em tanques. Lesões na pele e escamas constituem local de entrada de patógenos (BARCELLOS, SOUZA, WOEHL, 2000). A sedação também pode ser aplicada em manejos rápidos para extrusão de gametas, vacinação, coleta de sangue e pesagem (SNEDDON, 2012).

No entanto, para procedimentos invasivos como intervenções cirúrgicas, por exemplo, níveis profundos de depressão no SNC são necessários a fim de preservar o bem-estar do animal e facilitar o manuseio. Anestésicos também são importantes durante este tipo de operação para atuar na prevenção da dor, a qual pode levar a um aumento nos níveis de cortisol e consequentemente reduzir o apetite e aumentar a susceptibilidade a doenças ou predação (SNEDDON, 2012, 2015). Porém, deve-se estabelecer o protocolo ideal que delimite tempo de exposição à determinada concentração para evitar o aprofundamento irreversível da anestesia. Assim, um dos requisitos primordiais para a utilização de anestesia em peixes é a determinação de concentrações ótimas do fármaco de acordo com a finalidade do procedimento (PARK et al., 2008).

Uma vez terminado o manejo para o qual o animal foi anestesiado, possibilita-se ao mesmo recobrar sua atividade normal, processo este denominado recuperação da anestesia. A exposição ao fármaco é interrompida, ocorrendo a gradual reversão dos estágios observados durante a indução (NEIFFER, STAMPER, 2009).

1.3.2 Fatores que influenciam a anestesia de peixes

Além do fármaco e do tempo de exposição, a eficácia e a segurança da anestesia em peixes também dependem de variáveis biológicas e ambientais (BURKA et al., 1997; SNEDDON, 2012). Espécie, idade, estágio de desenvolvimento, tamanho, razão entre área branquial e peso, condição corporal, sexo e maturidade sexual são algumas das variáveis relativas ao animal que devem ser consideradas (OLSEN, EINARSDOTTIR, NILSSEN, 1995; COYLE, DURBOROW, TIDWELL, 2004; GOMES et al., 2011). Fêmeas em fase reprodutiva, por exemplo, com grande quantidade corporal de lipídios, podem permanecer sob efeito anestésico por períodos prolongados devido à lenta eliminação de anestésicos

lipossolúveis (ROSS, ROSS, 2008). Condições ambientais como temperatura e concentração de oxigênio devem ser analisadas antes do procedimento anestésico (KING et al., 2005; GOMES et al., 2011). Temperaturas altas podem acelerar a indução e a recuperação, enquanto situações de hipóxia podem retardar ou até mesmo impedir a recuperação da anestesia. Além disso, oscilações bruscas nos parâmetros de qualidade da água podem estressar os peixes, o que influi na difusão do anestésico devido a alterações na taxa ventilatória (ZAHL et al., 2009; SNEDDON, 2012).

1.3.3 Anestésicos e vias de administração em peixes

A anestesia de peixes é mais comumente realizada por meio de banhos de imersão, sendo o fármaco dissolvido na água e absorvido principalmente pelas brânquias e em menor quantidade através da pele (SNEDDON, 2012). Para a recuperação, os animais são transferidos para aquários livres de substância anestésica (NEIFFER, STAMPER, 2009).

Os anestésicos sintéticos geralmente empregados por esta via são o metanosulfonato de tricaína (MS222), a benzocaína, a quinaldina e o fenoxietanol (ROSS, ROSS, 2008; MARICCHIOLO, GENOVESE, 2011). Estes compostos possuem indiscutível ação anestésica e são amplamente empregados em peixes. Contudo, podem induzir toxicidade em animais e manuseadores (VELISEK et al., 2007; ROSS, ROSS, 2008). Não existem leis acerca do uso de anestésicos para peixes no Brasil, sendo as recomendações do *Food and Drug Administration* (FDA, 2006), órgão governamental norte-americano, normalmente seguidas. No entanto, o único anestésico aprovado pelo FDA para uso nestes animais, o MS222, não é comercializado no Brasil e as taxas de importação são altas e restringem sua aquisição (ROUBACH, GOMES, VAL, 2001). Há também anestésicos sintéticos utilizados para anestesiar peixes por via parenteral, como a lidocaína, o pentobarbital, a quetamina, o metomidato e o propofol. Porém, são empregados em menor escala que os anestésicos dissolvidos na água devido à necessidade de contenção física individual para a aplicação (FLEMING et al., 2003; MULCAHY et al., 2011). No entanto, há trabalhos descrevendo o uso de alguns destes fármacos na anestesia de peixes em banho de imersão (SMALL, 2003; PEYGHAN et al., 2008; GRESSLER et al., 2012; ANSCHAU et al., 2014; OSTRENSKY, PEDRAZZANI, VICENTE, 2015).

Os fármacos supracitados são classificados em duas categorias: anestésicos locais e anestésicos gerais. Entretanto, quando absorvidos via brânquias nos peixes, todos agem sistemicamente e inibem a transmissão do sinal neuronal do sistema nervoso periférico ao

SNC (SUMMERFELT, SMITH, 1990; ZAHL, SAMUELSEN, KIESSLING, 2012). Os anestésicos locais atuam através do clássico bloqueio de canais de Na^+ (MAMA, STEFFEY, 2003). Já os anestésicos gerais podem, por exemplo, inibir receptores excitatórios (nicotínico de acetilcolina, 5-hidroxitriptamina e *N*-metil-D-aspartato) e/ou potencializar receptores inibitórios ($GABA_A$ e glicina) (STEFFEY, 2003). São exemplos de anestésicos locais o MS222, a benzocaína e a lidocaína e de anestésicos gerais a quinaldina, o fenoxietanol, o pentobarbital, a quetamina, o metomidato e o propofol.

Anestésicos obtidos de extrativos vegetais representam uma alternativa aos sintéticos para anestesia de peixes em banho de imersão. O mentol, o óleo de cravo e o eugenol, além de óleos essenciais extraídos de folhas de plantas como *Aloysia triphylla*, *Aloysia gratissima*, *Ocimum gratissimum*, *Lippia alba, Myrcia sylvatica*, *Curcuma longa* e *Hesperozygis ringens* estão entre os produtos testados (FAÇANHA, GOMES, 2005; VIDAL et al., 2008; PEREIRA-DA-SILVA et al., 2009; CUNHA et al., 2010a; BECKER et al., 2012; BENOVIT et al., 2012, 2015; HELDWEIN et al., 2012; SILVA et al., 2012, 2013a,b; SALBEGO et al., 2014; TONI et al., 2014, 2015, SACCOL et al., 2016).

1.3.3.1 Metanosulfonato de tricaína

O metanosulfonato de tricaína (metanosulfonato do 3-amino benzoato de etila), ou MS222, é atualmente o anestésico mais utilizado na anestesia de peixes em banho de imersão, sendo aprovado nos Estados Unidos (FDA, 2006), Canadá (HEALTH CANADA, 2011) e Reino Unido (VETERINARY MEDICINES DIRECTORATE, 2011) para algumas espécies de peixes utilizadas no consumo humano. Sua eficácia na anestesia de peixes foi reconhecida em 1920 e desde então é recomendado para uso em uma grande variedade de espécies (PALMER, MENSINGER, 2004; KIESSLING et al., 2009; ZAHL et al., 2010; MATSCHE, 2011). É também utilizado para anestesia de anfíbios e outros animais ectotermos (HIKASA et al., 1986; CORDOVA, BRAUN, 2007).

Apesar da absorção do MS222 ocorrer em grande parte pelas brânquias, a pele também pode configurar um local de entrada deste fármaco em algumas espécies. Uma vez na circulação sanguínea, este derivado da benzocaína é rapidamente distribuído para o resto do organismo. A biotransformação é hepática e renal e a eliminação é principalmente branquial (HUNN, ALLEN, 1974; BURKA et al., 1997; SATO et al., 2000).

O MS222 bloqueia canais de Na^+ voltagem-dependentes nas membranas neuronais (FRAZIER, NARAHASHI, 1975; BUTTERWORTH, STRICHARTZ, 1990; BURKA et al.,

1997; BAI et al., 2003). A alta lipossolubilidade deste fármaco permite uma fácil penetração no interior da célula para ligar-se com canais de Na^+ e inibir a entrada deste no nervo, bloqueando a despolarização, assim como os demais anestésicos locais (HUNN, ALLEN, 1974; BUTTERWORTH, STRICHARTZ, 1990). Consequentemente, a excitabilidade da membrana neuronal fica limitada (HUNN, ALLEN, 1974; HIKASA et al., 1986; BUTTERWORTH, STRICHARTZ, 1990). Frazier e Narahashi (1975) também descreveram a supressão da corrente de K^+ na membrana neuronal como efeito adicional do MS222.

De acordo com Gilderhus e Marking (1987), o MS222 apresenta grande qualidade anestésica por promover rápidas indução e recuperação. Concentrações acima de 500 mg L^{-1} podem apresentar natureza ácida quando usadas em sistemas com alcalinidade abaixo de 50 mg/L $CaCO_3$, o que requer o uso de agentes neutralizantes a fim de evitar a acidemia metabólica (FERREIRA, SCHOONBEE, SMIT, 1984; MURRAY, 2002; PALMER, MENSINGER, 2004). Alterações nos níveis de hemoglobina, hematócrito e íons plasmáticos, bem como hipoxemia, hipercapnia, acidose respiratória e hiperglicemia foram relatados em peixes anestesiados com MS222 (IWAMA, MCGEER, PAWLUK, 1989; SLADKY et al., 2001; DAVIS, GRIFFIN, 2004). O MS222 apresenta boa margem de segurança, mas água mole e temperatura elevada podem alterá-la, especialmente em peixes jovens (GILDERHUS, MARKING, 1987; ROUBACH, GOMES, VAL, 2001; COYLE, DURBOROW, TIDWELL, 2004).

A *American Veterinary Medical Association* (AVMA, 2013) indica o MS222 para a eutanásia de peixes. A resolução Nº 1000 (2012) do Conselho Federal de Medicina Veterinária e o Conselho Nacional de Controle de Experimentação Animal (CONCEA, 2013) também recomendam o uso do MS222 para eutanasiar estes animais em banho de imersão ou por aspersão branquial, apesar de o fármaco não estar disponível para aquisição no país.

1.3.3.2 Propofol

O propofol (2,6 di-isopropilfenol) é um anestésico geral intravenoso utilizado mundialmente na anestesia de mamíferos, aves, répteis e anfíbios, além de ser amplamente empregado na medicina humana (BORIN, CRIVELENTI, LIMA, 2010; HIPPALGAONKAR, MAJUMDAR, KANSARA, 2010; MULCAHY et al., 2011). Alguns trabalhos relatam o uso de propofol, nas concentrações de 2,5 a 7,5 mg/kg, para anestesia intravenosa em peixes (FLEMING et al., 2003; MILLER, MITCHELL, HEATLEY, 2005;

NEIFFER, STAMPER, 2009). Além disso, sua aplicação por via intravenosa é recomendada pelo CONCEA (2013) para a eutanásia de peixes.

Peyghan et al. (2008) empregaram propofol (2 a 10 mg/L) na anestesia da carpa capim *Ctenopharyngodon idella* em banhos de imersão e observaram sua eficácia. Na busca por um produto disponível no Brasil, com preço acessível e com potencial para ser utilizado na anestesia de peixes em banhos de imersão, o estudo realizado por Gressler et al. (2012) testou propofol (2,5 a 12 mg/L) em jundiás cinzas e albinos e também obteve resultados satisfatórios. Subsequentemente, o propofol foi investigado como anestésico dissolvido na água em douradinho *Carassius auratus* (GHOLIPOURKANANI, AHADIZADEH, 2013), coregono-lavareda *Coregonus lavaretus* (GOMULKA et al., 2014), tilápia nilótica (VALENÇA-SILVA et al., 2014) e lambari-do-rabo-amarelo *Astyanax altiparanae* (OSTRENSKY, PEDRAZZANI, VICENTE, 2015), promovendo anestesia em todas as espécies.

O propofol é um alcifenol, sendo, portanto, altamente lipossolúvel e insolúvel na água. Para aumentar sua hidrossolubilidade, está disponível como emulsão aquosa, a qual possui coloração opaca esbranquiçada (HIPPALGAONKAR, MAJUMDAR, KANSARA, 2010). É extensamente captado pelo SNC, resultando em induções rápidas, e prontamente redistribuído do cérebro para outros tecidos, sendo em seguida removido do plasma pelo metabolismo (FLEMING et al., 2003). Devido à depuração corpórea total do propofol ser rápida e exceder o fluxo sanguíneo hepático, acredita-se que outros tecidos, além deste, estejam envolvidos na sua metabolização. É metabolizado primariamente por conjugação com glicuronídeos e sulfatos, resultando em metabólitos inativos que são excretados na urina (SIMONS et al., 1991; VEROLI et al., 1992). O fármaco não acumula no organismo e sua margem de segurança é ampla (MILLER, MITCHELL, HEATLEY, 2005; MITCHELL, 2009).

A ação do propofol é exercida por modulação positiva do complexo-receptor subtipo A do neurotransmissor inibitório GABA, $GABA_AR$ (FOWLER et al., 2001; KRASOWSKI et al., 2002; SAARI et al., 2011). O receptor está presente no cérebro e na medula espinhal, locais críticos para a perda de consciência e mobilidade, respectivamente (BARNARD et al., 1998). Consiste em um canal de Cl^- circundado por cinco subunidades homólogas (α, ß, γ, ε e π). O canal de Cl^- se abre após a liberação do GABA nos neurônios pré-sinápticos, estando envolvido na regulação da vigilância, tensão muscular e memória (BAI et al., 2001; KRASOWSKI et al., 2002; MOHLER, FRITSCHY, RUDOLPH, 2002; CAMPAGNA-SLATER, WEAVER, 2007; SAARI et al., 2011). Além de baixas concentrações de propofol aumentarem a condutância do Cl^-, altas concentrações do fármaco são capazes de ativar a

corrente iônica através do $GABA_AR$ mesmo na ausência do neurotransmissor (BELELLI et al., 1996; SANNA et al., 1999; YAMAKURA et al., 2001). De acordo com Richardson et al. (2007), o sítio de ligação do propofol no $GABA_AR$ é uma tirosina no segmento M4 da subunidade ß2. No entanto, outros autores postulam que o sítio de ligação do fármaco no receptor é uma metionina no segmento M3 da subunidade ß2 (KRASOWSKI et al., 2001; BALI, AKABAS, 2003).

Alguns estudos descrevem efeitos depressores sobre os sistemas cardiovascular e respiratório em decorrência do uso de propofol, principalmente quando usado em altas concentrações. No entanto, os riscos são mínimos para animais saudáveis (GOODCHILD, SERRAO, 1989; ILKIW et al., 1992). No peixe *Chiloscyllium plagiosum*, por exemplo, a anestesia com propofol não alterou a respiração nem os batimentos cardíacos (MILLER, MITCHELL, HEATLEY, 2005). E na carpa capim, os efeitos da anestesia com propofol sobre a atividade cardíaca e o pH e gases sanguíneos foram considerados mínimos (PEYGHAN et al., 2008).

A característica neutralidade do propofol faz com que a ocorrência de patologias em consequência do seu uso seja praticamente nula, sendo muito baixa sua toxicidade para mamíferos (SHORT, BIFILAR, 1999; SHORT, YOUNG, 2004). Em tilápias nilóticas, não foram observados efeitos genotóxicos e mutagênicos em decorrência da exposição a baixas concentrações do anestésico (VALENÇA-SILVA et al., 2014). Além disso, o efeito antioxidante do propofol é extensamente descrito (BAO et al., 1998; XIA, GODIN, ANSLEY, 2004; VOLTI et al., 2006; KUANG et al., 2008; PILAR et al., 2008). O fármaco apresenta semelhanças na sua estrutura molecular com a vitamina E (α-tocoferol), um importante antioxidante natural. A estrutura fenólica do propofol o torna um potente capturador de elétrons de radicais livres, formando um intermediário relativamente estável, impedindo assim a oxidação de importantes componentes celulares (RUNZER et al., 2002; VOLTI et al., 2006).

1.3.3.3 Óleo essencial de Aloysia triphylla

A *Aloysia triphylla* é um arbusto de folhas perenes que pode crescer até 3 m de altura. Pertence à família Verbenaceae e é nativo da América do Sul (Brasil, Argentina, Chile, Uruguai e Peru), onde tem sido amplamente empregada na medicina popular (VALENTÃO et al., 2002; ZAMORANO-PONCE et al., 2006). Entre seus nomes comuns estão cidrão, cidró, erva-luísa (JORGE et al., 2007), bela-aloísia, doce-lima e limonete (CUNHA, SILVA,

ROQUE, 2003). Suas sinonímias são: *A. citriodora* Ortega ex Pers.; *A. citriodora* Paláu; *A. sleumeri* Moldenke; *Lippia citriodora* H.B.K.; *L. citriodora* (Lam.) Kunth; *L. triphylla* (L'Her.) Kuntze; *Verbena citriodora* Cav.; *V. triphylla* L'Hér.; *Zapania citriodora* Lam. (CARNAT et al., 1999; VALENTÃO et al., 1999; PASCUAL et al., 2001; SANTOS-GOMES, FERNANDES-FERREIRA, VICENTE, 2005; DUKE, GODWIN, OTTESEN, 2008).

O óleo essencial de *A. triphylla* já foi avaliado para sedação e anestesia de invertebrados aquáticos e peixes. Em pós-larvas e sub-adultos de camarão branco *Litopenaeus vannamei*, o óleo promoveu sedação (20-50 µL/L) para transporte e anestesia (300 µL/L) satisfatórias, além de estimular o sistema de defesa antioxidante (PARODI et al., 2012). Em robalo-peva *Centropomus parallelus*, sedação (25-50 µL/L) e anestesia (100-300 µL/L) também foram verificadas; no entanto, quando utilizado para o transporte (24 h) dos animais nas concentrações de 10 e 20 µL/L, ocasionou mortalidade de 30 e 70%, respectivamente (PARODI et al., comunicação pessoal). O óleo também foi eficaz em promover sedação para transporte (30 a 50 µL/L) e anestesia (200 µL/L) de jundiás cinzas e albinos, mas induziu o aumento da concentração corporal de cortisol em jundiás albinos transportados com 40 e 50 µL/L (PARODI et al., 2014). No entanto, seu modo de ação ainda não foi elucidado.

A composição química da infusão desta planta apresentou grandes quantidades de compostos polifenólicos (675 mg/L), dentre os quais estão o verbascosídeo (400 mg/L) e a luteolina-7-diglucoronídeo (100 mg/L), além de 42 mg/L de óleo essencial, sendo o citral um dos constituintes do mesmo (CARNAT et al., 1999). Compostos polifenólicos têm sido descritos como capturadores de diversas espécies oxidantes (SHIMOI et al., 2000; BENAVENTE-GARCÍA et al., 2002). Valentão et al. (2002) também descreveram a *A. triphylla* como potente antioxidante por apresentar capturadores de ácido hipocloroso e radicais superóxido e hidroxil. De acordo com Alarcón et al. (2008), compostos polifenólicos de plantas podem reduzir os efeitos deletérios das espécies ativas de oxigênio (EAO) em diversos processos biológicos e patológicos. Em contrapartida, Dadé et al. (2009) afirmaram que *A. triphylla* apresenta baixa atividade antioxidante, apesar de ser rica em compostos polifenólicos, sugerindo que seria mais importante o tipo de composto polifenólico presente que a quantidade total do mesmo. Deve-se ressaltar que a composição química de plantas sofre influências das oscilações nos fatores climáticos. Logo, o horário do dia e a estação de colheita, entre outros fatores, determinam alterações nos constituintes das plantas. Da mesma forma, processamentos pós-colheita também acarretam variações na identificação dos componentes químicos de plantas (SCHWOB et al., 2004; BRANT et al., 2009).

1.4 MORFOLOGIA BRANQUIAL DE PEIXES

As brânquias dos peixes possuem uma grande área de contato com o meio externo, através da qual ocorrem processos essenciais para a manutenção da vida do animal, como respiração, alimentação, osmorregulação, equilíbrio ácido-base e excreção de produtos nitrogenados, além de absorção e excreção de fármacos (HUNN, ALLEN, 1974; PERRY, 1998; SAKURAGUI, SANCHES, FERNANDES, 2003; EVANS, PIERMARINI, CHOE, 2005). Em peixes teleósteos, as brânquias são formadas por quatro arcos branquiais situados ao lado da faringe, os quais são separados do meio externo pelos opérculos. Cada arco possui duas fileiras de filamentos branquiais, os quais contêm as lamelas (HIROSE et al., 2003). O fluxo de sangue através das lamelas é contracorrente com o fluxo de água entre elas, favorecendo as trocas entre os capilares sanguíneos e o ambiente aquático (FERNANDES, MAZON, 2003; EVANS, PIERMARINI, CHOE, 2005).

O epitélio das lamelas é contínuo com o do filamento. Porém, há variação nos tipos celulares encontrados em cada local (MORON, FERNANDES, 1996). O epitélio lamelar possui duas camadas de células, sendo a mais externa formada por células pavimentosas. Estas estão implicadas nas trocas gasosas e por isso estão em íntimo contato com a água, além de serem as mais numerosas; logo, o epitélio da lamela é denominado epitélio respiratório (PERRY, 1998). Internamente, há células indiferenciadas e pilares, sendo as últimas responsáveis pela sustentação dos espaços entre os vasos sanguíneos (MORON, ANDRADE, FERNANDES, 2009).

O epitélio do filamento, por sua vez, possui múltiplas camadas, as quais são formadas por células indiferenciadas, neuroepiteliais, mucosas, pavimentosas e de cloreto (BAILLY, DUNEL-ER, LAURENT, 1992; PERRY, 1997). As células de cloreto possuem ampla área de superfície e têm função primordial na osmorregulação (PERRY, 1997; HIROSE et al., 2003). As enzimas Na^+/K^+-ATPase e Ca^+-ATPase, além de trocadores e canais iônicos, estão presentes nas membranas basolateral e apical destas células, o que demonstra o papel ativo das mesmas no transporte iônico (FLIK et al., 1995; DANG et al., 2000; FERNANDES, MAZON, 2003; LINGWOOD, HARAUZ, BALLANTYNE, 2006; DYMOWSKA, HWANG, GOSS, 2013). Para tanto, uma grande quantidade de energia é necessária, a qual é fornecida pelas mitocôndrias que estão presentes em abundância neste tipo de célula. As células de cloreto são responsáveis pela absorção ativa de Na^+, Cl^- e Ca^+ em peixes de água doce, os quais perdem íons constantemente para o meio (PERRY, 1997). Se houver necessidade de aumentar a captação iônica, há proliferação de células de cloreto no epitélio do filamento, a

qual pode expandir para o epitélio lamelar dependendo da intensidade do estímulo (PERRY, 1998). Água mole, por exemplo, induz proliferação destas células no epitélio lamelar, o que resulta em comprometimento da capacidade respiratória (PERRY, WOOD, 1985). Ocorre um aumento na espessura do epitélio respiratório, ficando maior a distância de difusão gasosa entre o sangue e a água (GRECO et al., 1995).

Devido à sua localização e participação em funções vitais, as brânquias são consideradas excelentes biomarcadores, tanto para monitorar a saúde de ecossistemas como a do próprio peixe (WINKALER et al., 2001; FERNANDES, MAZON, 2003). Agentes estressores, como fármacos e poluentes, podem causar distúrbios hidrominerais e respiratórios, por exemplo, por influenciar diretamente na integridade da estrutura branquial, ou indiretamente, por ativarem a cascata hormonal de resposta ao estresse, o que promove alterações na permeabilidade e na morfologia das brânquias (BARTON, IWAMA, 1991; WENDELAAR BONGA, 1997; EVANS, PIERMARINI, CHOE, 2005). No entanto, as alterações morfológicas nas brânquias podem representar adaptações para que o peixe enfrente mudanças ambientais e conserve o balanço osmótico e hidroeletrolítico, sem comprometer o funcionamento tecidual (LAURENT, PERRY, 1991).

1.5 ESTRESSE OXIDATIVO EM PEIXES

O estudo da atividade antioxidante em peixes também fornece uma importante referência sobre o estado fisiológico destes animais. A vida aeróbica é essencialmente dependente de oxigênio em seu estado molecular, O_2 (MARTÍNEZ-ÁLVAREZ, MORALES, SANZ, 2005). Ao mesmo tempo, este também pode ser tóxico aos organismos aeróbicos, o que é referido como o “paradoxo da vida aeróbica”, uma vez que existe o risco permanente de estabelecimento de estresse oxidativo (DAVIES, 2000; AHMAD, PACHECO, SANTOS, 2004).

De acordo com Sies (1991), o estresse oxidativo ocorre quando há uma elevação sustentada na concentração das EAO acima de seus níveis fisiológicos. O excesso de EAO (ânion superóxido, peróxido de hidrogênio, radical hidroxil e oxigênio singlete) pode acontecer em situações que impliquem em diminuição dos níveis das defesas antioxidantes, em aumento na velocidade de produção das espécies ativas, ou em ambas, gerando um desequilíbrio entre a produção das EAO e dos sistemas de defesa antioxidante. As EAO são geradas endogenamente (principalmente nas mitocôndrias) como consequência direta do metabolismo do O_2 e também em situações não-fisiológicas, como a exposição da célula a

xenobióticos, que provocam a redução incompleta do O_2 (ROSS, MOLDEUS, 1991; CADENAS, DAVIES, 2000).

Os efeitos deletérios do estresse oxidativo causam primeiramente um dano celular reversível, o qual pode progredir para a irreversibilidade, e são dependentes de variáveis como o tipo de organismo, seu estado fisiológico e sua dieta. Esses efeitos incluem oxidação de macromoléculas (substratos oxidáveis) como proteínas, hidratos de carbono, DNA e RNA (podendo inclusive induzir mutações) e de componentes esteróides, bem como peroxidação de lipídios insaturados em membranas celulares. O resultado da lipoperoxidação são hidroperóxidos de lipídios instáveis, sendo os produtos de sua decomposição altamente reativos, constituindo uma ameaça à integridade celular. Além disso, esses produtos podem quebrar-se em radicais livres, os quais podem perpetuar o círculo destrutivo de reações em cadeia de peroxidação lipídica (HALLIWELL, 1992; PAVANATO, LLESUY, 2008).

Assim como os demais seres aeróbicos, os peixes apresentam sistemas de defesa antioxidante para proteger o organismo, desenvolvidos ao longo da escala evolutiva (MARTÍNEZ-ÁLVAREZ, MORALES, SANZ, 2005). Segundo Pavanato e Llesuy (2008), a função dos mecanismos antioxidantes é manter baixas as concentrações em estado estacionário das EAO, seja prevenindo sua formação ou capturando-as quando se formam. Há duas linhas de defesa antioxidante, sendo uma delas formada por substâncias não-enzimáticas, como a vitamina C, a vitamina E, o ácido úrico, a glutationa e os carotenóides. A outra engloba diversas enzimas que previnem a cascata de reações oxidantes, interceptando e inativando os intermediários reativos de oxigênio, bloqueando o ciclo de peroxidação lipídica. Essas enzimas são decisivas para contra-atacar a toxicidade do oxigênio quando o suprimento de outros componentes antioxidantes é escasso ou inexistente (AHMAD, PACHECO, SANTOS, 2004). Enzimas antioxidantes especialmente adaptadas, como a catalase (CAT) e a superóxido dismutase (SOD), bem como as enzimas dependentes de glutationa, a glutationa peroxidase (GPx) e a glutationa redutase (GR), já foram detectadas na maioria dos peixes (RUDNEVA, 1997).

Considerando estados de anóxia ou hipóxia, como os induzidos pela anestesia, Barry (1994) relatou que o momento mais crítico é a recuperação da normóxia, e não a redução de oxigênio propriamente dita. A reação à hipóxia mais comumente observada em peixes é o aumento das defesas antioxidantes, tanto enzimáticas quanto não-enzimáticas. Buzadzic et al. (1992) referiram-se a este processo como preparação para o estresse oxidativo. Estudos mostraram que, ao contrário do que ocorre em mamíferos, a anóxia ativa as defesas antioxidantes em peixes (VIG, NEMCSOK, 1989; LUSHCHAK et al., 2001; COOPER et al.,

2002; WILHEM FILHO et al., 2005; DI MARCO et al., 2008; DOLCI et al., 2013) e em outros ectotermos (HERMES-LIMA, STOREY, STOREY, 1998). Para Hochachka e Lutz (2001) e Hermes-Lima e Zenteno-Savín (2002), tal resposta detectada em peixes é considerada uma adaptação evolutiva, protegendo o organismo durante condições de escassez de oxigênio, o que faz com que o animal tolere o estresse oxidativo decorrente da reoxigenação tecidual. Em mamíferos, a reperfusão de órgãos isquêmicos gera a produção excessiva de EAO e induz peroxidação lipídica, oxidação proteica e dano ao DNA, devido a um baixo potencial antioxidante de órgãos anóxicos ou hipóxicos (LEFER, GRANDER, 2000; WHITE, SULLIVAN, DEGRACIA, 2000; HERMES-LIMA, ZENTENO-SAVÍN, 2002).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Este estudo objetiva utilizar fármacos sintéticos e naturais para sedar e anestesiar jundiás em banhos de imersão e investigar eficácia anestésica e alterações em indicadores morfofisiológicos, buscando-se alternativas com potencial para uso na piscicultura brasileira.

2.2 OBJETIVOS ESPECÍFICOS

1) Verificar o tempo de indução e recuperação da anestesia de jundiás com MS222 ou óleo essencial de *A. triphylla* e analisar *status* antioxidante, dano oxidativo, cortisol plasmático, hematócrito e balanço iônico em ambas as etapas da anestesia (Artigo 1);

2) examinar parâmetros hematológicos, morfológicos, bioquímicos e hidrominerais em jundiás sedados com propofol (Artigo 2); e

3) caracterizar a morfologia branquial e os níveis de pró-oxidantes e antioxidantes em jundiás sedados com propofol (Artigo 3).

3 ARTIGOS

Três artigos são apresentados nesta tese:

1) 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 (DOI 10.1111/are.12043).

2) Hematological, morphological, biochemical and hydromineral responses in *Rhamdia quelen* sedated with propofol (DOI 10.1007/s10695-014-9997-5).

3) Histological and antioxidant responses in *Rhamdia quelen* sedated with propofol (DOI 10.1111/are.12682).

O artigo 1, que relata os efeitos do tradicional anestésico sintético para peixes, MS222, e de um produto natural, o óleo essencial de *A. triphylla*, foi publicado no periódico *Aquaculture Research* (Qualis B3 nas Ciências Biológicas II) em 2014. O artigo 2 foi publicado no periódico *Fish Physiology and Biochemistry* (Qualis B2 nas Ciências Biológicas II) em 2015 e o artigo 3 também no *Aquaculture Research* (Qualis B3 nas Ciências Biológicas II) em 2015, sendo que ambos relatam o uso do propofol.

3.1 ARTIGO 1

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.

Running title: Responses to natural and synthetic anaesthesia

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Abstract

Responses to anaesthesia with essential oil (EO) of *Aloysia triphylla* (135 and 180 $mg.L^{-1}$) and tricaine methanesulfonate (MS222) (150 and 300 $mg.L^{-1}$) were assessed in silver catfish. Exposure to the anaesthetics elicited a stress response in the species. In the case of MS222, it was displayed as a release of cortisol into bloodstream, elevation in hematocrit and plasma ion loss. The EO presented cortisol blocking properties, but increased hematocrit and disturbances of hydromineral balance were observed. Liver antioxidant/oxidant status of EO and MS222 anesthetized silver catfish was also estimated. The synthetic anaesthetic induced lipoperoxidation, notwithstanding increased catalase contents, whereas the naturally occurring product was capable of preventing the formation of lipid peroxides, possibly due to combined actions of catalase and glutathione-S-transferase. Anaesthetic efficacy was also tested via induction and recovery times. Overall, the promising results obtained for the physiological parameters of the EO treated fish counterbalanced the slight prolonged induction time observed for 180 $mg.L^{-1}$. As for 135 $mg.L^{-1}$, both induction and recovery times were lengthy; despite that, the EO was able to promote oxidative protection and mitigate stress. None of the MS222 concentrations prompted such responses concomitantly.

Keywords: *Rhamdia quelen,* anaesthetic, MS222, *Aloysia triphylla*, physiological impact

Introduction

Silver catfish, *Rhamdia quelen*, is endemic to freshwater from southern Mexico to central Argentina. Its husbandry has been markedly spreading in southern Brazil. Hence, it is needed to explore alternative methods for managing stress to assure its welfare during routine manipulations (Baldisserotto 2009).

Physiological stress is a non-specific response composed of primary, secondary and tertiary changes. The primary component is a neuroendocrine hormonal phase characterized by stimulation of the hypothalamic-pituitary-interrenal (HPI) axis, culminating with catecholamines and corticosteroids release (Wendelaar Bonga 1997). Secondary responses comprise the effects trigged by such hormones on metabolism, reproduction and the immune system, the purpose being to help fish overcome stress (Mommsen, Vijayan & Moon 1999). Tertiary or whole-animal changes in performance can result from the primary and secondary responses and possibly affect survivorship (Barton 2002).

Proper application of anaesthesia is a handy tool in the management of captive fish. Nonetheless, anaesthetics may fail in suppressing stress-induced activation of the HPI axis (Iwama, McGeer & Pawluk 1989; Zahl, Kiessling Samuelsen & Olsen 2010). Evaluation of blood cells, blood chemistry and hormones, especially cortisol concentrations in plasma, are broadly used as stress response indicators in fish (Barton and Iwama 1991).

Fish antioxidant defense systems may as well be affected by anaesthesia. The slow respiratory rate during the anaesthetic procedure induces sustained hypoxia, what in turn activates enzymatic (e.g. catalase, superoxide dismutase and glutathione-S-transferase) and non-enzymatic (e.g. reduced glutathione) antioxidant defenses (Di Marco, Priori, Finoia, Petochi, Marino, Lemarié, Alexis, Alberti & Macciantelli 2008). The aim is to compensate for the oxidative stress that occurs within the early minutes of recovery during tissue

reoxygenation, and the accompanying boost in the generation of reactive oxygen species (ROS) (Buzadzic, Spasic, Saicic, Radojicic & Petrovic 1992). Polyunsaturated fatty acids, which are involved in the regulation of lipid metabolism, constitute a preferred target for ROS (Storey 1996). Lipid peroxidation is, thus, a helpful biochemical indicator of oxidative damage (van der Oost, Beyer & Vermeulen 2003).

Cedron, *Aloysia triphylla* (L'Hérit) Britton, is a shrub native to South America but has been introduced into North America and Europe (Santos-Gomes, Fernandes-Ferreira & Vicente 2005; Zamorano-Ponce, Morales, Ramosa, Sepúlveda, Cares, Rivera, Fernández & Carballo 2006). Citral (neral and geranial) and limonene are the most representative components of the essential oil (EO) of *A. triphylla*, to which many of the oil observed effects are attributed (Carnat, Carnat, Fraisse & Lamaison 1999; Oliva, Beltramino, Gallucci, Casero, Zygadlo & Demo 2010). A recent investigation in south Brazil proved the EO to be able to promote sedation and anaesthesia in fish (Patent nº PI 016090005905 - Heinzmann, Baldisserotto, Parodi, Cunha, Silva & Mallmann 2010) and it is also known for its antioxidant properties (Valentão, Fernandes, Carvalho, Andrade, Seabra & Bastos 2002). Its anaesthetic efficacy was validated in shrimp too (Parodi, Cunha, Heldwein, de Souza, Martins, Garcia, Wasielesky Junior, Monserrat, Schmidt, Caron Heinzmann & Baldisserotto 2012).

Tricaine methanesulfonate (MS222) is the most widely used immersion anaesthetic for fish and is currently approved by the U.S. Food and Drug Administration (FDA) for anaesthesia of some food fish species. It is not available for purchase in Brazil, where research on fish anaesthetics is recurrently conducted so as to provide the users with accessible alternatives. In this context, essential oils represent a promising line of study (Cunha, Barros, Garcia, Veeck, Heinzmann, Loro, Emanuelli & Baldisserotto 2010; Silva, Parodi, Reckziegel, Garcia, Bürger, Baldisserotto, Malmann, Pereira & Heinzmann, 2012).

The purpose of this investigation was to compare the physiological impact of immersion anaesthesia with EO *A. triphylla* and with MS222 on silver catfish physiology. Primary (cortisol plasmatic levels) and secondary (plasma electrolytes concentrations and haematocrit) features of stress response, as well as the antioxidant status, were measured in the fish. Induction and recovery times were also assessed.

Materials and methods

Drugs

Tricaine-S™ (tricaine methanesulfonate; Western Chemical Inc., Ferndale, WA, US) was acquired in the U.S. Trials were conducted at concentrations of 150 and 300 $mg.L^{-1}$, according to Gressler, Parodi, Riffel, da Costa & Baldisserotto (2012).

The EO was obtained from leaves of cedron shrubs grown in Frederico Westphalen (RS, Brazil) and harvested in spring, 2009. Extraction was performed by hydrodistillation in Clevenger modified apparatus for 3 h (European Pharmacopoeia 2007). A voucher specimen (SMDB No. 11169) was deposited in the herbarium of the Department of Biology (UFSM). The EO was stored at – 4ºC in amber glass bottles and its average density was approximately 0.9 $g.mL^{-1}$. The oil was previously diluted in ethanol (1:10) and used at concentrations of 135 and 180 $mg.L^{-1}$, which were established in a pilot study.

Animals

Grey silver catfish (95.63 ± 2.83 g and 22.12 ± 0.2 cm), regardless of sex, were obtained from a fish farm in Santa Maria, southern Brazil. The fish were housed indoors in a

semi-static system at the Laboratory of Fish Physiology (UFSM), where the experiments were performed. For the 7-day acclimation period, the specimens were placed in 250 L tanks with approximately 200 L of fresh water that was constantly aerated by means of 20 W pumps and airstones and fed to satiation once a day with commercial pellets for omnivorous fish (42% extruded crude protein; 4% fibrous matter; 14% mineral matter; 2.5% calcium). The study was approved by the Ethics Committee on Animal Experimentation of UFSM under registration n° 46/2010.

Experimental design

The experiment consisted of ten groups (N=8 for each group). For every concentration of anaesthetic tested, two different groups were formed: anaesthesia and recovery. After withholding food for 24 h, individual fish were transferred to the induction aquarium and staged during anaesthesia according to the criteria outlined in Schoettger and Julin (1967) (Table 1). When the fish were deemed to have reached stage 4, or after 30 min had elapsed, the length and weight of the subjects were rapidly measured. Fish in the anaesthesia groups had blood collected (maximum 30 seconds between removal of the anaesthetic bath and bleeding) before being euthanized. Livers were then dissected out. Fish in the recovery groups were allowed to recuperate individually in aquaria free of anaesthetic agents as soon as biometrics was performed. Recovery was determined to be the time needed to restore full equilibrium, normal breathing and swimming, and to regain responsiveness towards external stimuli. Once at that state, fish were bled, euthanized and livers excised. Induction and recovery times of the recovery groups were recorded. The immersion solutions were changed every four fish in an attempt to expose the specimens to the appropriate concentration of the

anaesthetics. Water in the recovery aquaria was also renewed after four fish had recuperated in it.

Animals in the control group were transferred from one aquarium to another, both with water free of anaesthetic substance, duplicating the handling described above for the other groups. A similar protocol was employed with the vehicle group, only the water in the first aquarium contained ethanol at the highest concentration used to dilute the oil. With that accomplished, fish in the control and vehicle groups were weighed, measured and blood was collected. Euthanasia preceded liver dissection.

Blood sampling in all of the groups was performed from the caudal plexus with heparinized sterile syringes. Samples were subsequently transferred to microcentrifuge tubes and centrifuged at 3000 xg for 10 min (Centrifuge 5804 R). Plasma was then stored for 72 h at – 25 °C awaiting hormone and ion analyses. Livers were kept at – 4 °C for 24 h pending preparation for prooxidant and antioxidant evaluation. Euthanasia was performed by section of the spinal cord.

The trials were conducted in 10 L aquaria filled to 20% of their capacity with continuously aerated fresh water. The water source for acclimation and trials was the same and its quality parameters were 20 ± 1.0 ºC, pH 7.2 ± 0.9 and dissolved oxygen 7.0 ± 0.7 $mg.L^{-1}$.

Determination of prooxidant and antioxidant levels

Tissue preparation

To determine the non-enzymatic antioxidant reduced glutathione (GSH), through thiol groups, liver samples were homogenized in trichloroacetic acid (TCA) 20% and centrifuged at

1110 xg in a refrigerated centrifuge for 10 min (Ellman 1959). For analysis of TBARS, CAT, SOD and GST portions of the livers were homogenized in a medium containing 120 mM KCl and 30 mM buffer phosphate (pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were then centrifuged at 1110 xg for 20 min, between 0 and 4°C to eliminate nuclei and cell debris. The obtained supernatants were frozen at −80°C awaiting analysis, which were completed within 30 days (Buege & Aust 1978).

Protein assay

Protein content of the homogenate was estimated spectrophotometrically at 625 nm by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as a standard.

Prooxidant assay

The end products of lipid peroxidation, such as malondialdehyde, were measured according to Buege and Aust (1978) in order to monitor TBARS. The supernatant was added to a pyrex tube containing TCA 10% and thiobarbituric acid (TBA) 0.67% and incubated at 100ºC for 45 min. The blend was allowed to cool on ice for 5 min and then centrifuged at 1000 xg for 15 min, in order to extract the resulting chromogen (Schiff's base). The absorbance of the organic phase was determined at 535 nm in spectrophotometer. Results are presented as nmol.mg protein^{-1}.

Antioxidant defenses assay

Catalase activity was assayed by measuring the decrease in the absorption at 240 nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide, as outlined in Boveris and Chance (1973). Results are reported as pmol.mg protein^{-1}.

Total SOD activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/sodium hydroxide (pH 10.2). The enzyme activity was expressed as SOD units.mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation, as described by Misra and Fridovich (1972).

Glutathione-S-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically at 340 nm according to Habig, Pabst & Jak (1974). The assay was performed using 100 mM potassium phosphate buffer, pH 6.5, with GSH and CDNB at a final concentration of 1 mM each. Activity was calculated from the changes in absorbance at 340 nm. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 pmol of CDNB with GSH per minute at 25°C. The enzymatic activity is expressed as pmol.min^{-1} mg protein^{-1}.

The method employed to determine thiol groups, an indirect measure of GSH, is based on GSH reacting with 5,5'-ditio-bis-2-nitrobenzoic (DTNB) acid. The final product formed is the yellow 2-nitro-5-mercapto-benzoic (TNB) acid. The samples were read spectrophotometrically at 412 nm (Ellman 1959). The content of thiol groups is expressed as µmol.mg protein^{-1}.

Cortisol determination

Cortisol was measured in duplicates, in the unextracted plasma samples, using commercially available EIA kits (EIAgenTM Cortisol, Adaltis Italy S.p.A). The specificity of

the test was evaluated by comparing the parallelism between the standard curve and serial dilutions in PBS (pH 7.4) of the plasma samples. The standard curve, constructed with human standards provided by the kit, ran parallel to that obtained using serial dilutions of silver catfish plasma. In the linear regression test, a high positive correlation (R2=0.9818) was observed between the curves. The inter- and intraassay coefficients of variation ranged from 9% to 12% and 6% to 9%, respectively.

Hematocrit determination

Just after sampling, an aliquot of blood was used to fill duplicate microhematocrit capillary tubes. Centrifugation was carried out at 10000 xg for 10 min and the percentage of packed red cells was obtained by means of a hematocrit (Htc) card reader.

Plasma ion levels determination

Plasma concentrations of Na^+ and K^+ were measured in appropriate diluted samples against known standards using flame photometry (Micronal B262). Chloride levels were assessed via the colorimetric method according to Zall, Fisher & Garner (1956).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). A Levene test was used to verify homogeneity of variances. The Kruskal-Wallis test, followed by multiple comparisons of mean ranks, was used for analysis of glutathione-S-transferase. All of the

other parameters were analyzed through a one-way ANOVA and Tukey's test. The analyses were made with Statistica software 7.0 ($P < 0.05$).

Results and discussion

Induction and recovery times with MS222 and EO *A. triphylla*

A higher concentration hastened induction of anaesthesia in the MS222 treated fish (Figure 1A), corroborating previous reports with the anaesthetic in *Acipenser oxyrinchus oxyrinchus* (Matsche 2011) and in *Pagellus bogaraveo* and *Seriola dumerilii* (Maricchiolo & Genovese 2011). The EO also showed anaesthetic activity concentration-dependent in silver catfish (Figure 1B). Exposure of the same species to EO *Lippia alba* similarly evinced such dependence (Cunha *et al.* 2010).

With both concentrations of EO, the fish took over 3 min to achieve stage 4, (Figure 1) which is the time recommended by earlier reports as ideal for fish anaesthetic induction (Gilderhus & Marking 1987; Park, Jo, Lee, Kim, Park, Hur, Yoo & Song 2003). These authors maintain it would mitigate hypoxemia and acute stress responses. The time to complete full anaesthesia at 150 $mg.L^{-1}$ MS222 was equally higher than that mentioned above (Figure 1). It also diverged from the results reported by Seigneur (1984) when testing an equivalent concentration of the anaesthetic in the same fish species, what could be due to differences in fish size and/or trademark of the anaesthetic (none of which were mentioned in the cited manuscript). In accordance with Gressler *et al.* (in press), the present study registered a considerably short time to anaesthetize the specimens at 300 $mg.L^{-1}$ MS222 (Figure 1). Ethanol alone showed no anaesthetic effect.

The highest concentration of EO and both concentrations of MS222 promoted recovery times within the 10 min (Figure 1) suggested as a desirable time for fish to return from anaesthesia (Gilderhus & Marking 1987; Park *et al*. 2003). When the concentration of the EO was raised, the fish recuperated faster (Figure 1). A similar pattern was displayed by the species when subjected to EO *L. alba* anaesthesia (Cunha *et al*. 2010). Recovery from anaesthesia was unaffected by MS222 concentration (Figure 1), as observed in *P. bogaraveo* and *S. dumerilii* (Maricchiolo & Genovese 2011). An opposite trend was reported in *A. oxyrinchus oxyrinchus* (Matsche 2011), with prolonged recovery as MS222 concentration increased. All of the fish recovered from anaesthesia within 30 min and no animal loss was registered in the course of the experiment.

The effect of an anaesthetic agent is strongly related to its pharmacokinetic properties (Zahl *et al*. 2010). MS222 is a salt and therefore presents water-soluble features (Carter *et al*. 2011). Essential oils, in turn, are mixtures of lipophilic components and thus have high affinity for fatty tissue (Zahl *et al*. 2010). Higher lipid solubility of a substance may also accelerate its passage through the blood-brain barrier, resulting in a more profound central effect and leading to insufficient respiration and gill circulation, what delays elimination through the gills (Hunn & Allen 1974). These data may partly explain the longer recovery times observed for the plant derived anaesthetic.

Prooxidant and antioxidant levels with MS222 anaesthesia

The liver is one of the main mitochondrial sites of ROS generation during reoxygenation (Wilhem Filho 2007). The present study found induction of lipid peroxidation in the liver of fish sampled after recovery from the highest MS222 concentration (Table 2). Reintroduction of oxygen into hypoxic/anoxic tissues is likely to cause oxidative stress if

there is an overproduction of ROS that is not counterbalanced by the antioxidant defenses (Storey 1996; Lushchak, Lushchak, Mota & Hermes-Lima 2001). Conversely, Lushchak, Bagnyukova, Lushchak, Storey & Storey (2005) observed an increase in TBARS levels in *Cyprinus carpio* liver already under hypoxia, which was persistently high in reoxygenation. Besides the effect triggered in the tissue by oxygen restoration, MS222 might also induce lipoperoxidation in the organ. In this context, Velisek, Stara, Li, Silovska & Turek (2011) reported an increase in ROS formation after exposure of *Oncorhynchus mykiss* to some anaesthetics, including MS222.

Superoxide dismutase was unaffected by MS222 anaesthesia in silver catfish (Table 2). Likewise, Velisek *et al*. (2011) did not observe any difference in hepatic SOD activity between *O. mykiss* in the control group and the specimens sampled immediately after MS222 anaesthesia. Nonetheless, Cooper, Clough, Farwell & West (2002) claimed that SOD activity should rise as oxygen concentration decreases in order to detoxify ROS, as observed by the authors in *Leiostomus xanthurus*.

The content of GSH did not change in silver catfish over the course of hypoxia and reoxygenation in MS222 trials (Table 2). This data corroborates previous findings in *C. carpio* anesthetized with the same agent (Lushchak *et al*. 2005).

Catalase is one of the three primary antioxidant enzymes involved in ROS removal, and its increase in oxygen-limiting conditions is rather anticipated (Cooper *et al*. 2002). At both concentrations of MS222, the “preparation to oxidative stress” concept (Buzadzic *et al*. 1992) could be observed in the greater CAT concentration in the fish under anaesthesia, especially at the highest concentration, in which the enzyme value during recovery was back to control levels (Table 2). Low oxygen availability also induced elevation of CAT in liver of *Carassius auratus* (Lushchak *et al*. 2001), while in *L. xanthurus* (Cooper *et al*. 2002) and *C. carpio* (Lushchak *et al*. 2005) it was not observed. Sampaio, Boijink, Oba, Santos, Kalinin &

Rantin (2008), in turn, reported that hypoxia decreased the enzyme concentration in liver of *Piaractus mesopotamicus*. Moreover, induction of the enzyme activity could be an attempt to mitigate the toxic effects induced by the anaesthetic (Velisek, Stara, Li, Silovska & Turek 2011). Nonetheless, CAT alone was not efficient enough to combat lipid peroxide generation in silver catfish recovered from the highest concentration of MS222 (Table 2).

Catalase is fundamentally linked to SOD and their activities are dependent upon one another (Wilhem Filho, Giulivi & Boveris 1993). Consequently, correlative changes in both enzymes levels would be expected, providing the first defense system against oxygen toxicity (Pandey, Parvez, Sayeed, Haque, Bin-Hafeez & Raisuddin 2003). Nevertheless, SOD induction was not observed in this study (Table 2), in agreement with previous reports by Hermes-Lima & Storey 1998 and Cooper *et al*. 2002. The latter authors suggested that this enzyme could respond better to a long-term stimulus in opposition to an instantaneous one (experimental scale).

It is known that GST is a detoxification enzyme with a key role in the removal of reactive compounds, thereby protecting cells against ROS-induced damage (Rodriguez-Ariza, Dorado, Peinado, Pueyo & Lopez-Barea 1991). The current investigation observed induction of GST during reoxygenation (recovery group) with the lowest MS222 concentration (Table 2). In opposition to the “preparation to oxidative stress” theory, the pattern displayed by GST indicates a more conventional response to stress, which is an elevation of the enzyme activity as a consequence of the oxidative stress undergone during the hypoxic-normoxic transition (Halliwell & Gutteridge 1989). Along with CAT, GST stimulation may have prevented elevation of TBARS in the fish recovered from the lowest concentration of the anaesthetic (Table 2). The consumption of GST observed during anaesthesia with 150 mg.L^{-1} MS222 and anesthesia and recovery with 300 mg.L^{-1} MS222 could be due to its readily inactivation by

oxydation. In contrast, the activity of the enzyme in liver of *C. carpio* was unaffected by both hypoxic exposure and aerobic recovery (Lushchak *et al*. 2005).

Cortisol, hematocrit and plasma ion levels with MS222 anaesthesia

Studies exploring MS222 in different fish species showed the anaesthetic concentrations of 150-200 mg.L^{-1} as capable of preventing changes in plasma cortisol levels (Holloway, Keene, Noakes & Moccia 2004; Sink, Strange & Sawyers 2007; Matsche 2011), while lower, sedating ones, would elicit rather than inhibit cortisol response (Iwama *et al*. 1989; Maricchiolo & Genovese 2011). Silver catfish, nonetheless, requires a higher concentration of the anaesthetic to prevent cortisol release, as its levels increased in the plasma of the fish both during anaesthesia and after recovery at 150 mg.L^{-1} MS222 (Table 3). At 300 mg.L^{-1} MS222, however, cortisol secretion was not induced (Table 3). In this context, it is convenient to bring up the 6 min difference in induction time between the concentrations of MS222 tested in this study, indicating that time may play an important part in the stress response mechanism, besides the anesthetic itself, its concentration and the fish species, among a combination of other abiotic/biotic aspects.

Stress-related changes in hematological parameters occur within seconds or minutes after fish are disturbed (Barton & Iwama 1991). Indeed, the acute model employed in this study triggered an increase in Htc in MS222 anesthetized fish (Table 3). The same was observed by Sampaio *et al*. (2008) in *P. mesopotamicus* subjected to hypoxia. The mechanism accountable for Htc increase in the current study is unknown, but the prompt response supports the hypothesis of splenic contraction, causing an increase in red blood cell count in an attempt to absorb more oxygen to meet the increased demand (Sampaio *et al*. 2008). In *O. mykiss* (Iwama *et al*. 1989) and *S. dumerilii* (Maricchiolo & Genovese 2011), increase in Htc

levels also arose from anaesthetic hypoxia. Nonetheless, Htc values in anesthetized *Oncorhynchus tshawytscha* (Cho & Heath 2000) did not differ from pre-induction values.

A progressive Cl^- extrusion from control to anaesthesia and from anaesthesia to recovery was seen at the highest concentration of MS222 (Table 3). Sodium levels also decreased from fish sampled in the anaesthetic state to those allowed to recover (Table 3). Gills are the primary site of net Na^+ and Cl^- transport through the activity of chloride cells, playing a vital role in maintaining balance of the internal osmotic environment (Breves, Hirano & Grau 2010). Stress typically entails an increase in oxygen uptake through the gills to support enhanced respiratory demands, by means of increased branchial blood pressure and respiratory surface (Wendelaar Bonga 1997). Such responses necessarily involve increased diffusive ion and water movements across the gill, what is called the osmorespiratory compromise (Randall, Baumgarten & Malyusz 1972; Gonzalez & MacDonald 1994). No alterations were observed in K^+ levels (Table 3).

Prooxidant and antioxidant levels with EO *A. triphylla* anaesthesia

Lipoperoxidation process did not vary between groups in the EO trials (Table 4). When assessing *Leporinus elongatus* exposure to different O_2 concentrations for a prolonged period of time, Wilhem Filho, Torres, Zaniboni-Filho & Pedrosa (2005) observed lower TBARS levels in fish exposed to severe hypoxia compared to moderate hypoxia and normoxia, thus reflecting the elevated environmental O_2 availability in the latter two groups and its tissue consumption. The authors also indicated that outcome as a consequence of the maintenance and/or increase in antioxidants examined in the same study. In the present work, higher CAT and GST levels and maintenance of SOD and GSH levels were observed in the fish sampled under anaesthesia compared to control (Table 4), what may be in agreement with

the proposed by the authors above-mentioned. Up-regulation of CAT and GST was probably responsible for preventing the increase in lipid peroxidation damage during recovery, avoiding, thus, injurious levels.

Citral, one of the major constituents of EO *A. triphylla*, is a monoterpenoid which induces glutathione-S-transferase class π, $GSTP_1$ (Edris 2007). In citral-treated rat hepatocyte cells, $GSTP_1$ has shown to increase total GST activity (Nakamura, Miyamoto, Murakami, Ohigashi, Osawa & Uchida 2003). The hypothesis that citral was at least partially accountable for GST induction in the EO assessments (Table 4) may be speculated, along with the idea that antioxidant defenses are exacerbated during physiological states (e.g. anoxia/hypoxia) in which oxygen free radical production is reduced (Lushchak *et al*. 2005).

Cortisol, hematocrit and plasma ion levels with EO *A. triphylla* anaesthesia

The EO prevented cortisol release in silver catfish (Table 5). Likewise, silver catfish anesthetized with EO *L. alba* displayed no changes in plasma cortisol levels after deep anaesthesia (Cunha *et al*. 2010). There are no studies assessing how *A. triphylla* components influence plasma cortisol dynamics in fish. Nonetheless, it may be believed that there is a blockage of sensory information transmission to the hypothalamus, thus not triggering the hormonal cascade, as suggested by Iversen, Finstad, McKinley & Eliassen (2003) when discussing clove oil preventive behaviour upon cortisol release in *S. salar*. In contrast, Cho and Heath (2000) found elevated cortisol levels in *O. tshawytscha* both during clove oil anaesthesia and after recovery. Maricchiolo and Genovese (2011) similarly observed an elevation of cortisol levels in *S. dumerilii* under clove oil anaesthesia.

Increased Htc levels were detected in silver catfish under anaesthesia with the EO (Table 5). Maricchiolo & Genovese (2011) also observed that Htc levels of clove oil

anesthetized *S. dumerilii* were significantly higher than controls. Nonetheless, no alterations in Htc were reported for *O. tshawytscha* anesthetized with clove oil (Cho & Heath 2000).

Silver catfish presented higher Na^+ plasma levels during anaesthesia with the lowest concentration of EO (Table 5). Baldisserotto, Chippari-Gomes, Lopes, Bicudo, Paula-Silva, Almeida-Val & Val (2008) reported the same pattern of ion flux in hypoxic *Serrasalmus eigenmanni*, suggesting that hypoxia would decline gill ventilation and oxygen uptake, minimizing ion loss through the gills. On the other hand, there was a decrease in plasma K^+ levels during anaesthesia with the highest concentration of the anaesthetic, and no changes were observed for plasma Cl^- during anaesthesia or recovery (Table 5). Similar plasma Cl^- levels were registered for control and clove oil anesthetized sunshine bass (*Morone chrysops* x *Morone saxatilis*), but a significant decrease was observed after recovery (Davis & Griffin 2004).

Conclusions

Fish anaesthesia is a complex procedure. It must be well orchestrated in order to achieve its purposes, the most important of which being to spare fish health. Tricaine methanesulfonate has been considered the prototypical immersion anaesthetic for fish since the 1920s, especially for being able of promoting fast induction and recovery times. Nonetheless, studies as the present one have been demonstrating that its physiological impact could be highly detrimental to fish, what supports research on new anaesthetic drugs. This preliminary investigation on the essential oil of *Aloysia triphylla* showed promising results, with stimulation of the antioxidant capacity and prevention of lipoperoxidation, and also suppression of cortisol release. Its anaesthetic effect was not observed within the preconized time limit, but it was still a reasonable one. In sum, a rapid induction time may not be so

crucial in determining the potential of a given anaesthetic, and other aspects involving its use ought to be taken into account.

Acknowledgements

Funding for this research was provided by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul/Programa de Apoio a Núcleos de Excelência (FAPERGS/PRONEX), Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Tecnológico (CNPq). Authors also thank CNPq for providing research fellowship for B. Baldisserotto and CAPES for graduate fellowships to L.T. Gressler, A.P.K. Riffel, T.V. Parodi, E.M.H Saccol and G. Koakoski. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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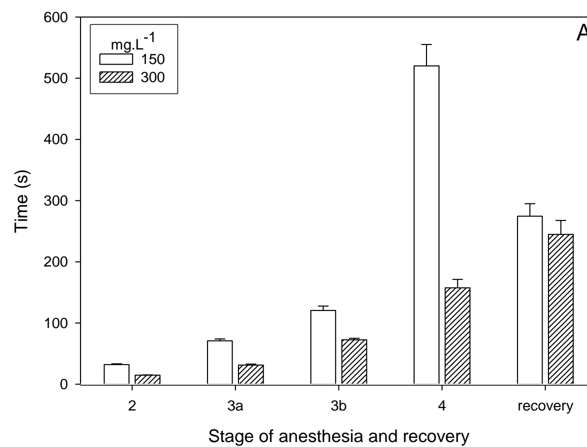
Figure legend

Figure 1 - Time required for induction and recovery from anaesthesia in silver catfish with 150 and 300 mg.L^{-1} MS222 (A) and 135 and 180 mg.L^{-1} EO *Aloysia triphylla* (B). Stages are according to Schoettger and Julin (1967). Maximum observation time was 30 min. Time to reach each stage is in seconds (s). N=8 for each concentration.



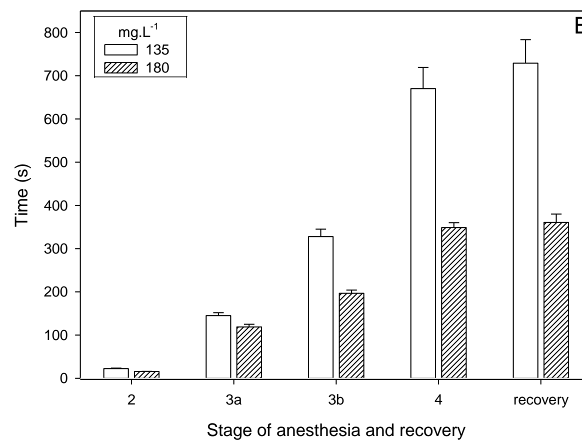


Table 1 - Stages of anaesthesia in fish (Schoettger and Julin, 1967).

Stage	Description	Behavioural response
1	Light sedation	Partial loss of reaction to external stimuli
2	Deep sedation	Partial loss of equilibrium, no reaction to external stimuli
3a	Total loss of equilibrium	Fish usually turn over but retain swimming ability
3b	Total loss of equilibrium	Swimming ability stops but responds to pressure on the caudal peduncle
4	Anaesthesia	Loss of reflex activity, no reaction to strong external stimuli
5	Medullary collapse (death)	Respiratory movement ceases (death)

Table 2 - Oxidative parameters in liver of silver catfish anesthetized with MS222 (mean ± SEM).

Groups/ Parameters	TBARS (nmol.mg protein^{-1})	CAT (pmol.mg protein^{-1})	SOD (units.mg protein^{-1})	GST (pmol.min^{-1} mg protein^{-1})	GSH (µmol.mg protein^{-1})
Control	0.37±0.08	3.21±0.40	8.31±0.65	1.85±0.30	0.033±0.006
150 mg.L^{-1} MS222					
Anaesthesia	0.46±0.09	5.62±0.35*	8.87±0.47	0.47±0.10*	0.050±0.018
Recovery	0.85±0.21	6.1±0.26*	9.66±0.71	3.09±0.53^{+}	0.032±0.007
300 mg.L^{-1} MS222					
Anaesthesia	0.38±0.08	5.24±0.45*	8.85±0.50	0.90±0.17*	0.036±0.003
Recovery	1.43±0.35*$^{+}$	4.74±0.43	9.61±0.60	0.87±0.11*	0.042±0.007

* indicates significant difference from control and $^{+}$ significant difference from anaesthesia in the respective concentration by one-way ANOVA and Tukey test or Kruskal-Wallis test and multiple comparisons of mean ranks ($P < 0.05$). N=8 for each concentration group.

Table 3 - Cortisol, hematocrit and plasma ions in silver catfish anesthetized with MS222 (mean ± SEM).

Groups/ Parameters	CORTISOL ($ng.mL^{-1}$)	Htc (%)	Cl^- (mmol)	Na^+ (mmol)	K^+ (mmol)
Control	17.74±3.17	25.13±1.12	160.24±3.98	120.27±7.15	2.27±0.41
150 $mg.L^{-1}$ MS222					
Anaesthesia	26.24±1.50*	30.25±1.54*	148.47±6.06	119.48±7.58	2.39±0.44
Recovery	26.15±1.37*	28.0±0.77	144.57±3.53	138.99±12.25	1.69±0.33
300 $mg.L^{-1}$ M222					
Anaesthesia	19.15±2.97	31.94±1.26*	147.15±3.05*	134.69±3.53	2.15±0.25
Recovery	22.59±2.10	27.63±2.01	135.88±2.38*+	93.27±12.72+	2.89±0.30

* indicates significant difference from control and + significant difference from anaesthesia in the respective concentration by one-way ANOVA and Tukey test ($P < 0.05$). N=8 for each concentration group.

Table 4 - Oxidative parameters in liver of silver catfish anesthetized with EO *A.triphylla* (mean ± SEM).

Groups/ Parameters	TBARS ($nmol.mg\ protein^{-1}$)	CAT ($pmol.mg\ protein^{-1}$)	SOD ($units.mg\ protein^{-1}$)	GST ($pmol.min^{-1}\ mg\ protein^{-1}$)	GSH ($\mu mol.mg\ protein^{-1}$)
Control	0.37±0.08	3.21±0.40	8.31±0.65	1.85±0.30	0.033±0.006
Vehicle	0.57±0.11	4.11±0.56	8.26±0.48	1.25±0.15	0.024±0.006
135 $mg.L^{-1}$ EO					
Anaesthesia	0.34±0.07	6.06±0.98*	8.01±0.73	4.90±1.09*	0.028±0.004
Recovery	0.41±0.14	4.4± 0.44	9.74±1.30	2.48±0.42	0.021±0.005
180 $mg.L^{-1}$ EO					
Anaesthesia	0.49±0.10	9.12±2.28*	10.02±1.20	5.85±1.27*	0.033±0.006
Recovery	0.36±0.09	4.6± 0.59	10.27±1.20	2.11±0.7	0.028±0.002

* indicates significant difference from control by one-way ANOVA and Tukey test or Kruskal-Wallis test and multiple comparisons of mean ranks ($P < 0.05$). N=8 for each concentration group.

Table 5 - Cortisol, hematocrit and plasma ions in silver catfish anesthetized with essential oil (EO) of *Aloysia triphylla* (mean ± SEM).

Groups/ Parameters	CORTISOL ($ng.mL^{-1}$)	Htc (%)	Cl^- (mmol)	Na^+ (mmol)	K^+ (mmol)
Control	17.74±3.17	25.13±1.12	160.24±3.98	120.27±7.15	2.27±0.41
Vehicle	26.6±2.05	25.63±0.33	145.19±5.06	131.35±4.11	2.13±0.37
135 $mg.L^{-1}$ EO					
Anaesthesia	14.46±2.15	32.89±1.88*	163.29±3.19	145.50±4.75*	1.87±0.37
Recovery	18.53±2.30	21.12±1.64$^+$	154.73±3.46	139.44±6.28	2.64±0.56
180 $mg.L^{-1}$ EO					
Anaesthesia	11.0±2.31	30.81±0.73*	144.52±2.78	134.21±4.51	1.19±0.21*
Recovery	19.3±3.36	26.94±2.20	153.59±7.51	125.72±5.88	3.10±0.21$^+$

* indicates significant difference from control and $^+$ significant difference from anaesthesia in the respective concentration by one-way ANOVA and Tukey test or Kruskal-Wallis test and multiple comparisons of mean ranks ($P < 0.05$). N=8 for each concentration group.

3.2 ARTIGO 2

Hematological, morphological, biochemical and hydromineral responses in *Rhamdia quelen* sedated with propofol

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Abstract

Rhamdia quelen responses to propofol sedation were examined. The purpose was to investigate whether propofol would be a suitable drug to be used in fish transport procedures. Fish were exposed to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h in 40 L tanks, simulating open transport systems. Results indicated that the intermediate concentration of propofol was able to prevent cortisol release. It also preserved the stability of some hematological (hematocrit, RBC count, hemoglobin, MCV, MCH and MCHC) morphological (RBC area), biochemical (glucose, glucose, lactate, total protein, ammonia, urea, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase) and hydromineral (Na^+, Cl^- and K^+ plasma levels) indicators of stress. The highest concentration of the sedative also blocked cortisol secretion; nonetheless, it prompted mild physiological changes such as increase in hemoglobin concentration. Results suggested that sedation with propofol at the concentration of 0.4 mg L^{-1} is suitable for *R. quelen* transport.

Keywords transport; stress; haematology; morphology; biochemistry

Introduction

The transportation segment of the fish farming system undoubtedly requires operational expertise and knowledge on the physiology of the subjects. Buin et al. (2013) stated that mortality occurred not only during transport but also and most importantly after the fish had been delivered to the recipient location. The addition of sedative/anesthetic substances to the water of transport has been employed in an attempt to reduce fish death arisen from transport-mediated stress (Robertson et al. 1988; Ross et al. 2007; Becker et al. 2012; Benovit et al. 2012). The purpose is to induce a calming or sedative state during the procedure so that the perception of the stressful event is minimized and its side-effects reduced (Iwama et al. 1989).

The stress response in fish is an adaptive mechanism characterized by a cascade of physiological alterations that constitute a three phase pattern (Wendelaar Bonga 1997). Firstly, upon stress, the neuroendocrine system releases catecholamines and corticosteroids into the blood stream. The presence of these stress hormones in the circulatory system precipitates subsequent responses related to energy requirements, like increases in blood glucose and lactate levels, and variation in plasma electrolytes concentrations, among others. If the second responses are extreme or sustained, the process culminates with whole-animal changes which compromise metabolism, reproductive output and disease resistance, and may ultimately lead to mortality (Barton and Iwama 1991; Wendelaar Bonga 1997; Maricchiolo and Genovese 2011).

Despite the purpose of the use of an anesthetic being to mitigate stress, a common report is that the substance itself may pose as a stressor, thus activating the stress response mechanism (Thomas and Robertson 1991; Sladky et al. 2001; Bolasina 2006). According to Zahl et al. (2012), the unwanted side effects of an anesthetic such as respiratory acidosis and osmotic stress reduce the welfare of the fish and, therefore, caution should be taken when such agents are used. Another point to consider is that the efficacy of a given anesthetic depends on variables such as the intensity of the stressor, the fish species, its developmental stage and the environmental conditions (Rotllant et al. 2001; King et al. 2005).

In view of such considerations, the viability of using propofol as a sedative for juvenile silver catfish *Rhamdia quelen* (Quoy and Gaimard 1824) transport was investigated. This anesthetic has recently been proven effective for immersion anesthesia of the same fish species (Gressler et al. 2012a). The experiment was performed in a laboratory controlled setting in order to verify the sole effect of the anesthetic upon the physiology of the species

through the analyses of hematological, morphological, biochemical and hydromineral indicators of stress.

Methods

Animals

Juvenile grey silver catfish (*n* = 90; mean ± S.E. body mass = 91.44 ± 1.98 g; mean ± S.E. total length = 20.66 ± 0.15 cm) were acquired from a fish farm in Santa Maria city, southern Brazil, and housed at the Laboratório de Fisiologia de Peixes (LAFIPE) at Universidade Federal de Santa Maria (UFSM). Acclimation lasted seven days and was performed in 250 L tanks (15 fish/tank) in a semi-static system with constantly aerated dechlorinated well water (200 L/tank) at 21.5 ± 0.08 ºC, pH 7.45 ± 0.13 and dissolved oxygen 8.04 ± 0.26 mg L^{-1} (mean ± S.E.). The water was renewed every second day and the fish were fed commercial pellets for omnivorous fish (42% extruded crude protein; 4% fibrous matter; 14% mineral matter; 2.5% calcium) once a day. All procedures were conducted with the approval of the Ethics Committee on Animal Experimentation of the UFSM (registration n° 67/2012).

Drug

Propofol (Propotil 1%; BioChimico; www.biochimico.com.br) was commercially acquired. A pilot study based on literature-derived values (Gressler et al. 2012a) was performed and two low concentration levels of propofol were established to be used in sedative baths: 0.4 and 0.8 mg L^{-1}. These concentrations were able to induce up to stages 2 and 3a, respectively, as described by Schoettger and Julin (1967).

Experimental design

Twenty-four hours after the last feeding the fish were subjected to one of the following concentrations of propofol: 0 (control), 0.4 or 0.8 mg L^{-1}. Each treatment was further divided into an exposure time of 1, 6 and 12 h, reproducing short, medium and long transport procedures respectively. For every concentration/time combination, 10 fish were tested (two replicates of five fish each).

The trials were performed in 40 L tanks filled to 50 % of their capacity with the same water used in the acclimation tanks. The proper anesthetic concentration was dispersed in the water if that was the case. The experimental setting simulated transportation in tanks. Nonetheless, in order to guarantee that any observed effect would arise from the anesthetic only, care was taken to prevent common transport interferences such as decline in dissolved oxygen, build-up of ammonia, accumulation of carbon dioxide and reduction in pH from happening. Therefore, loading density was low, constant aeration was provided and the experiment was carried out under controlled environmental conditions at LAFIPE. Water parameters in the experimental tanks were: 21.8 ± 0.12 ºC, dissolved oxygen 6.5 ± 0.24 mg L^{-1} (Yellow Springs Instruments, Yellow Springs, OH, USA; model Y55), pH 7.4 ± 0.06 (Hanna Instruments, Woonsocket, RI, USA; model HI 8424), total ammonia 0.14 ± 0.02 mg L^{-1} (Verdouw et al. 1978) and un-ionized ammonia 0.002 ± 0.001 mg L^{-1} (Colt 2002).

Fish were hand-transferred to the trial tanks and kept under the confined experimental conditions for the assigned period. Once exposure time had elapsed, fish were individually removed from the tanks and a 2 mL blood sample was immediately taken from the caudal peduncle with heparinized sterile syringes. Biometrics was also performed and fish were euthanized by sectioning the spinal cord.

Whole blood analyses

Hematocrit was measured in microcapillary tubes centrifuged at 10000 Xg for 10 min and reading was performed with the aid of a hematocrit card reader. Total red blood cells (RBC) count was determined with a Neubauer hemocytometer (Tavares-Dias et al. 2002). The concentration of hemoglobin was assayed by the cyanmethemoglobin method using a spectrophotometer (Brow 1976). The indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Wintrobe (1934). Blood smears were prepared and then air-dried, fixed in methanol and stained with May-Grünwald (Rosenfeld 1947). Subsequently, with the aid of an image analyzer microscope, ten high-power fields were randomly selected on each blood smear and morphometry of six RBC was observed in each of these fields (Benfey et al. 1984; Dorafshan et al. 2008). All of the morphometric analyzes were performed using the Zeiss Axio Vision System with Remote Capture 4.7 Rel DC - Cannon Power shot G9.

Plasma analyses

The remaining whole blood was placed into microcentrifuge tubes and spun at 3000 Xg for 10 min. The obtained plasma was collected in microtubes and stored at -25 ºC for further analyses.

EIA kits (EIAgenTM Cortisol, Adaltis Italy S.p.A) were used to measure cortisol in unextracted plasma samples. Test specificity was assessed through comparison of the parallelism between the standard curve and serial dilutions of the samples in PBS (pH 7.4). The standard curve ran parallel to the one achieved with serial dilutions of *R. quelen* plasma. A high positive correlation (r^2 = 0.9818) was observed between the curves in the linear regression test. Inter- and intra-assay variation coefficients ranged from 9 % to 12 % and 6 % to 9 % respectively. Glucose, total protein, urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using kits by Analisa (www.goldanalisa.com.br). Analyses of lactate and alkaline phosphatase (AP) were determined in Labtest kits (www.labtest.com.br). Ammonia was quantified according to Verdouw et al. (1978). Concentrations of Na^+ and K^+ were measured in appropriate diluted samples against known standards using flame photometry (Micronal B262). Chloride levels were assessed via the colorimetric method (Zall et al. 1956).

Statistical analyses

The experimental variables were three propofol concentrations (0, 0.4 and 0.8 mg L^{-1}) and three exposure durations (1, 6 and 12 h). Data are presented as the mean ± standard error (S.E.). The Bartlett test was used to evaluate normality and the Levene test was applied to verify homogeneity of variances. Cortisol analyses were made through the Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Nemenyi test. The remaining parameters were analyzed through a two-way ANOVA and Tukey's test. The Statistica software 7.0 (Stat Soft. Inc., wwwstatsoft.com) was used to make the analyses, and differences were considered significant at $P < 0.05$.

Results

Whole blood analyses

At 0.4 mg L^{-1} propofol the hematocrit was lower at 6 compared to 12 h while at 0.8 mg L^{-1} propofol the hematocrit was lower at 1 h than at the remaining times. The level of this blood index was lower at 0.4 than at 0 and 0.8 mg L^{-1} propofol in fish exposed for 6 h. The concentration of hemoglobin was significantly greater within 6 h of exposure to the highest concentration of the anesthetic. Statistical evidence did not identify any effect on RBC, MCV, MCH or MCHC (Table 1). Propofol exposure reduced the nucleus of the cells (Table 2).

Plasma analyses

The level of cortisol in control group progressively declined from 1 to 12 h. The hormone concentration increased at 6 and 12 h in comparison with 1 h at 0.4 mg L^{-1}. The level of cortisol was greater at 6 than at 1 h exposure to 0.8 mg L^{-1} propofol; the group sampled at 12 h showed higher and lower concentrations of the hormone compared to 1 and 6 h respectively. Cortisol gradually reduced from the highest to the lowest concentration of the anesthetic at 1 h. At 6 h cortisol was higher at 0.8 than at 0 and 0.4 mg L^{-1} propofol. The level of the hormone was significantly higher at 0.4 than at 0 and 0.8 mg L^{-1} propofol in fish exposed for 12 h. Glucose concentration did not vary between groups. The content of lactate was significantly lower at 0.8 mg L^{-1} propofol after 1 and 6 h of exposure (Table 3). Protein, ammonia and urea were not significantly affected by the treatments (Table 3). The activity of AP showed an increase in fish sampled at 12 h after exposure to 0.8 mg L^{-1} propofol. No statistically significant alterations were observed in ALT or AST (Table 3). At 0 mg L^{-1} propofol significantly higher Cl^- levels were observed in the group sampled at 12 h compare to 6 h. The level of Na^+ increased after exposure to 0.4 mg L^{-1} propofol for 12 h and decreased at the same sampling time at the highest concentration. There was a significant rise in the level of K^+ in fish exposed to 0.8 mg L^{-1} propofol for 12 h (Fig. 1). Survival during the experiment was 100 %.

Discussion

Whole Blood Analyses

Hematocrit, RBC, Hemoglobin, MCV, MCH and MCHC

Propofol has been described as capable of decreasing ventilatory drive as well as cardiac output and contractility in mammals (Grouds et al. 1985; Pagel and Warltier 1993). Nevertheless, the results obtained for the hematological indices show that the anesthetic produced only mild hemodynamic changes in *R. quelen*, most prominently in the hemoglobin content. The values of RBC, MCV, MCH and MCHC, in turn, were preserved. The absence of alterations in these indices following anesthesia agrees with previous results (Velisk et al. 2005a, b) but diverges from others (Tort et al. 2002; Bolasina 2006; Gomulka et al. 2008; Sudagara et al. 2009; Pádua et al. 2012). Wells and Weber (1990), for instance, observed evidence of RBC swelling through low MCHC after anesthesia. Despite the statistical difference found for hematocrit, its range as well as the range of RBC, MCV and MCHC compares favorably with the results previously described as normal for *R. quelen* (Tavares-Dias et al. 2002). The content of hemoglobin, however, is slightly higher. Barcellos et al. (2003) also reported similar baseline values for *R. quelen* hematocrit, RBC, hemoglobin and MCHC, but not for MCV.

As in the present investigation, Tort et al. (2002), Bressler and Ron (2004) and Filiciotto et al. (2012) similarly noted a decrease in hematocrit percentage as a result of anesthesia. Nevertheless, increased level of this blood index following anesthetic administration is most commonly reported (Thomas and Robertson 1991; Olsen et al. 1995; Gomulka et al. 2008; Sudagara et al. 2009; Maricchiolo and Genovese 2011; Gressler et al. 2012b; Pádua et al. 2012). Elevated hematocrit percentage may occur due to plasma volume reduction, hypoxia, and a combination of RBC swelling and/or release by the spleen as a response to acute stress mediated by catecholamines (Davidson et al. 2000; Tort et al. 2002). In this study, in turn, the reduction in hematocrit may have been an adaptive response without major physiological significance especially because RBC number remained unchanged (Franklin et al. 1993). No influence of anesthesia on hematocrit has also been described (Cho and Heath 2000; Wagner et al. 2003; Velisek et al. 2005a, b).

The values found for hemoglobin concentration at 0.8 mg L^{-1} propofol after 6 h exposure may indicate that in this group there was a transient requirement for increased blood oxygen-carrying capacity. It was probably achieved by the movement of water from primary to secondary circulation systems, resulting in increased content of hemoglobin (Wells and Weber 1990; Franklin et al. 1993). The capacitance response is a rapid means of preserving oxygen delivery to tissues under hypoxic challenge, which in this case was mostly likely a result of the reduced gill ventilation during sedation or anesthesia, as previously related (Soivio et al. 1977; Molinero and Gonzalez 1995; Sudagara et al. 2009). Moreover, besides

the above-mentioned changes in RBC size or number, which may also be accountable for elevation in hemoglobin, as well as in hematocrit, Speckner et al. (1989) proposed that fish erythrocytes still synthesize hemoglobin while circulating in the peripheral blood. Thus, the increased levels of hemoglobin may reflect enhanced synthesis during low oxygen availability. In line with most of the results observed for hemoglobin in this study, previous works also described no effect of anesthesia on its concentration (Velisek et al. 2005a, b; Pádua et al. 2012).

RBC morphometry

The RBC are the most abundant cells in fish blood and their number and size represent the capacity of oxygen transportation (Fukushima et al. 2012). The RBC area obtained by means of morphometric analyses remained the same throughout the groups, what confirms that the use of propofol did not trigger major changes in the blood oxygen-carrying capacity besides the sole effect seen in the hemoglobin content for one specific experimental group. Morphometry is a valuable and accurate tool for obtaining fish RBC measurements without the interference of other variables, as in the case of MCV. But literature on it is still scarce, hence the lack of physiological evidence as to justify the reduction in the RBC nucleus of anaesthetized silver catfish.

Plasma analyses

Cortisol, glucose and lactate

One hour after being subjected to tank transfer, the fish in the control group showed the highest level of cortisol obtained in this investigation, 40.35 ± 4.85 ng mL^{-1}. Though not so high, such value is within the range reported in literature for the post-stress cortisol level in fish (Pickering and Pottinger 1989; Barton and Iwama 1991), and the 1 h peak is in agreement with similar responses observed by Auperin et al. (1997), Wagner et al. (2003), Bolasina (2006), Barcellos et al. (2012) and Koakoski et al. (2012). After a considerable decline 6 h within the experiment, at 12 h the level of cortisol was lowered to 19.12 ± 1.97 ng mL^{-1}. Similar values were obtained for unstressed *R. quelen* (Barcellos et al. 2006). Recovery to resting cortisol levels following an acute stressor of moderate intensity within 6 h was previously observed (Robertson et al. 1988; Pickering and Pottinger 1989). Barcellos et al.

(2012) and Koakoski et al. (2012) noticed that 4 h was sufficient to restore baseline pre-stress cortisol concentrations in *R. quelen* submitted to an active chase with a pen net for 60 sec.

A different response was observed when the fish were under propofol sedation. Although a significant difference was detected between the first sampling point and the two latter ones at 0.4 mg L^{-1} propofol, the level of cortisol was maintained within the pre-stress range throughout the testing. As for the highest concentration of the anesthetic, a low level of the hormone was found at 1 h, with a gradual increase in the following measurements. So, in comparison to control group, both sedative concentrations of the anesthetic influenced the dynamics of cortisol and prevented the hormone rise, maintaining the baseline value along the assessment time. Davis and Griffin (2004), Small (2004) and Gressler et al. (2012b) also observed that administration of anesthetics prevented cortisol increase in fish. In opposition to what was demonstrated by the current results, some authors state that a low concentration may actually act as a stressor because the level of nervous depression does not mitigate certain physiological stress responses (Robertson et al. 1988; Iwama et al. 1989; Olsen et al. 1995; Maricchiolo and Genovese 2011).

It is well accepted that catecholamines and corticosteroids inhibit glycogen synthesis and stimulate gluconeogenesis in order to fuel homeostatic mechanisms activated during exposure to stressors (Wendelaar Bonga 1997; Sladky et al. 2001). Furthermore, Polakof et al. (2012) cited that fish glycaemia increases as a result of treatment with exogenous cortisol, the presence of stressors and exposure to anesthetics, among other conditions. Indeed, preceding works observed hyperglycemia along with a rise in cortisol when some fish species were subjected to similar challenges (Rotllant et al. 2001; Barcellos et al. 2003; Wagner et al. 2003; Bressler and Ron 2004; Davis and Griffin 2004; Maricchiolo and Genovese 2011; Filiciotto et al. 2012). Nonetheless, Cho and Heath (2000), Davidson et al. (2000), Iversen et al. (2003), Bolasina (2006) and Matsche (2011) did not find significantly higher levels of glucose in association with cortisol elevation. Likewise, in the present study the increased cortisol in control group did not alter carbohydrate metabolism, most likely because of the low magnitude of the corticosteroid response. All groups had glucose levels within the baseline concentration reported earlier for unstressed *R. quelen* (Barcellos et al. 2003).

Although plasma glucose did not show the classic stress-induced catabolic response, the higher lactate observed at 1 and 6 h at 0 mg L^{-1} propofol in comparison with the highest concentration of the anesthetic showed the provision of a rapid energetic resource following the handling stress when sedation was not present. However, the low levels of the metabolite suggest that there was an initial activation of anaerobic metabolism contributing to ATP

supply, but without deficit in oxygen or glycidic resources. Rotllant et al. (2001) and Small (2004) also described stress-related responses of lactate, what happened along with a rise in glucose. Stressful conditions are typically associated with elevated plasma lactate concentrations, for the anaerobic state caused by stress results in muscle glycogen and lactate breakdown, with some of the lactate being released into circulation (Barton and Iwama 1991). In opposition to the present findings, anesthetics have been accountable for lactate rises (Soivio et al. 1977; Molinero and Gonzalez 1995; Olsen et al. 1995; Iversen et al. 2003; Wagner et al. 2003). As Iwama et al. (1989) explained, lactate increases in blood when insufficient oxygen is available for aerobic cell metabolism, what could be due to reduced ventilation and circulation, common side-effects of several anesthetics.

Total protein, ammonia and urea

Conceição et al. (2012) stated that, depending on the type of stressor imposed to fish, cortisol may have an effect on protein and amino acids metabolism. In the current research, the level of total protein was not affected by the experimental conditions, ranging from 4.73 ± 0.27 to 5.78 ± 0.24 g dL^{-1}. This range is in accordance with the values previously described for unstressed *R. quelen* (Barcellos et al. 2003). Davidson et al. (2000), Velisek et al. (2005a), Congleton (2006) and Pádua et al. (2012) also found no interference of handling stress or anesthesia on plasma protein levels. Barcellos et al. (2003), on the other hand, detected an effect of harvesting on total protein, indicating the possible use of such compound as substrate for the gluconeogenesis observed in the study. Laidley and Leatherland (1988) and Matsche (2011), in turn, registered a significant increase in plasma protein in fish subjected to anaesthesia comparing to control; the latter authors indicated this shift as a result of RBC destruction.

Ammonia accounts for the greatest fraction of nitrogenous waste in teleost fish, followed by urea (Kajimura et al. 2004). Both waste products were examined but none presented any difference between the groups, corroborating the observed absence of stress-induced protein utilization in this study. Nonetheless, in spite of finding elevated total protein level in plasma of anaesthetized fish, Gomulka et al. (2008) observed decreased ammonia concentration in the same subjects, owing the lower levels of the compound to a reduced metabolic rate. Instead, Velisek et al. (2005b) and Velisek et al. (2009) found higher concentrations of ammonia after anesthesia even though plasma protein remained unchanged.

AP, ALT and AST

Propofol has hepatic as well as extra-hepatic clearance routes in mammals (Mather et al. 1989; Matot et al. 1993). In fish, nonetheless, there are no studies assessing propofol metabolism, but the increased AP within 12 h of exposure to 0.8 mg L^{-1} propofol may be due to hepatic hyperactivity in order to metabolize the drug. In keeping with this work, some investigations indicated no changes in AST or ALT following anesthesia (Velisek et al. 2005a; Velisek et al. 2009). Congleton (2006), however, found that anesthesia did not affect AP, but induced AST and ALT elevation. Differently, increased ALT activity, decreased AP and unchanged AST (Gomulka et al. 2008), and decreased AST (Velisek et al. 2005b) were noted after anesthetic exposure. Barcellos et al. (2003) reported increased AP and AST in stressed *R. quelen*, indicating these elevations as a result of the regulation of hepatic metabolism promoted by the increased cortisol levels, what was not observed in this study. The latter authors also found no changes in ALT, in consistence with the present findings.

Cl^-, Na^+ and K^+

Hydromineral disturbance typically arises from stress in fish (Barton and Iwama 1991; Wendelaar Bonga 1997). At 0 mg L^{-1} propofol the concentration of Cl^- was significantly higher at 12 compared to 6 h, what may have been due to a shift of Cl^- from intracellular to extracellular space (McDonald and Robinson 1993). By contrast, Cl^- levels did not alter in stressed fish (Auperin et al. 1997).

Following propofol administration there were slight changes in Na^+ and K^+ concentrations which were related to both efflux and influx. Some authors state that anesthesia triggered a decline in Cl^- (Davis and Griffin 2004), Cl^- and Na^+ (Gressler et al. 2012b) and K^+ (Davidson et al. 2000) levels. Enhanced respiratory demands may arise from anesthesia, and, along with increased oxygen uptake, there is increased diffusive ion and water movements across the gill (Becker et al. 2012). On the other hand, anesthesia was associated with increased concentrations of Na^+ and K^+ (Sladky et al. 2001), Cl^- and Na^+ (Matsche 2011) and K^+ (Soivio et al. 1977; Congleton 2006). Unchanged levels of Cl^- and Na^+ (Congleton 2006) and K^+ (Matsche 2011; Gressler et al. 2012b) have also been observed in studies assessing the effects of anesthesia on osmoregulation.

Conclusion

The findings of this study suggest that the use of propofol at the concentration of 0.4 mg L^{-1} is suitable for *R. quelen* transport. No major or irreversible damage was observed through the evaluated indices, what implies that the anesthetic preserved the physiology of the fish during short, medium and long time exposure.

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Figure legend

Figure 1 - Plasma levels of Cl^- (A), Na^+ (B) and K^+(C) in *Rhamdia quelen* subjected to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h. Values are mean±S.E. Different upper case letters indicate significant difference between times within the same concentration and different lower case letters indicate significant difference between concentrations at a given sampling time by two-way ANOVA and Tukey's test.

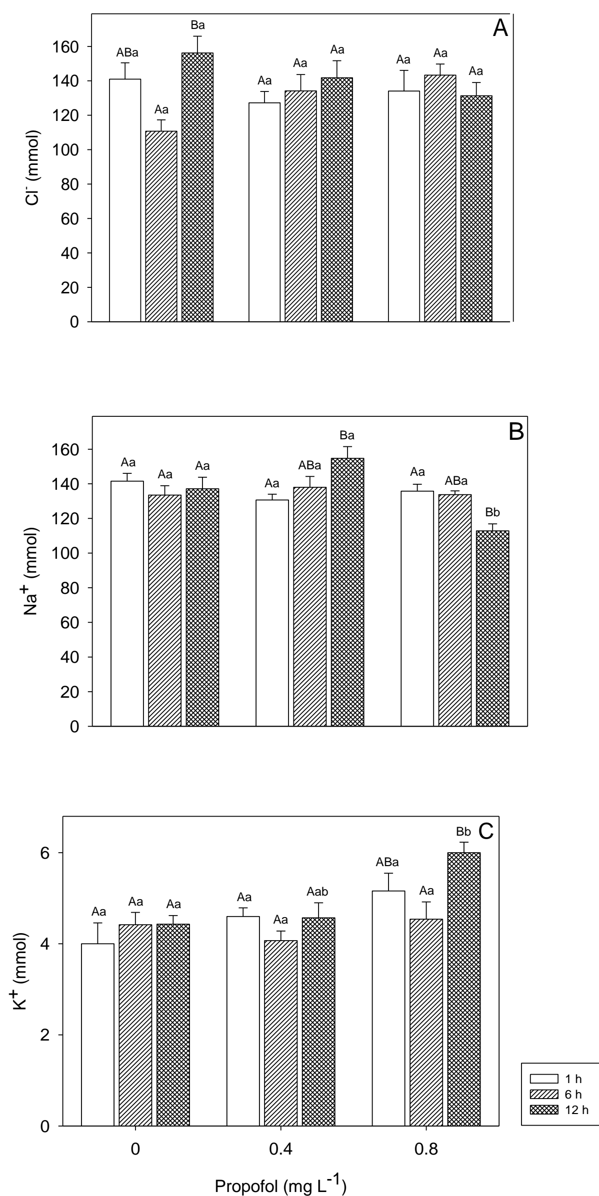

Table 1 Hematological parameters in *Rhamdia quelen* subjected to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h.

Groups/ Parameters	Hematocrit (%)	RBC (10^6 μL^{-1})	Hemoglobin (g dL^{-1})	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})
0 mg L^{-1} propofol						
1 h	28.8±1.08^{Aa}	2.23±0.17	7.19±0.60^{Aa}	125.68±9.82	32.27±1.53	25.49±2.54
6 h	32.0±0.85^{Aa}	2.22±0.16	8.15±0.56^{Aab}	137.81±8.19	37.60±2.09	25.85±2.17
12 h	29.3±0.47^{Aa}	2.46±0.13	7.30±0.60^{Aa}	121.43±5.67	30.51±2.82	24.95±1.89
0.4 mg L^{-1} propofol						
1 h	29.8±1.14^{ABa}	2.20±0.16	7.26±0.60^{Aa}	141.23±9.66	33.55±2.31	24.52±1.97
6 h	27.8±1.07^{Ab}	1.70±0.16	6.19±0.63^{Aa}	145.72±9.03	33.35±1.62	22.49±2.26
12 h	32.9±0.86^{Ba}	2.17±0.12	8.19±0.41^{Aa}	156.97±10.60	38.69±2.57	25.15±1.54
0.8 mg L^{-1} propofol						
1 h	26.1±0.39^{Aa}	2.24±0.19	6.94±0.31^{Aa}	114.84±9.72	33.53±3.70	26.56±0.99
6 h	32.5±1.25^{Ba}	2.25±0.13	9.93±0.59^{Bb}	150.50±13.95	42.08±1.98	31.01±2.22
12 h	31.6±1.16^{Ba}	2.27±0.16	7.30±0.70^{Aa}	146.28±12.46	32.79±2.24	23.38±1.78

Values are mean ± S.E. Different upper case letters indicate significant difference between times within the same concentration and different lower case letters indicate significant difference between concentrations at a given sampling by two-way ANOVA and Tukey's test. No significant differences were observed in RBC, MCV, MCH or MCHC.

Table 2 Red blood cell morphometry in *Rhamdia quelen* subjected to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h.

Groups/ Parameters	area (μm^2)	minor axis (μm)	major axis (μm)	nucleus area (μm^2)	nucleus minor axis (μm)	nucleus major axis (μm)
0 mg L^{-1} propofol						
1 h	123.31±2.90	11.11±0.13	14.19±0.13	19.18±0.64^{Aa}	4.63±0.07^{Aa}	5.38±0.09^{Aa}
6 h	115.88±2.68	10.68±0.10	14.33±0.16	17.37±0.55^{Aa}	4.24±0.07^{Ba}	5.33±0.08^{Aa}
12 h	116.24±2.56	10.70±0.17	14.08±0.14	18.37±0.83^{Aa}	4.42±0.11^{ABa}	5.39±0.11^{Aa}
0.4 mg L^{-1} propofol						
1 h	119.60±1.78	10.98±0.08	14.10±0.14	13.98±0.58^{Ab}	3.86±0.10^{Ab}	4.71±0.08^{Ab}
6 h	114.04±3.13	10.58±0.11	13.94±0.24	13.55±0.38^{Ab}	3.76±0.05^{Ab}	4.71±0.08^{Ab}
12 h	121.40±2.50	11.13±0.10	14.06±0.20	13.32±0.39^{Ab}	3.78±0.05^{Ab}	4.61±0.07^{Ab}
0.8 mg L^{-1} propofol						
1 h	113.95±2.29	10.66±0.15	13.80±0.17	15.00±0.54^{Ab}	4.05±0.08^{Ab}	4.83±0.08^{Ab}
6 h	119.06±1.75	10.92±0.10	14.80±0.12	14.11±0.42^{Ab}	4.77±0.10^{Bc}	4.77±0.10^{Ab}
12 h	119.18±1.55	10.99±0.08	14.01±0.12	12.98±0.27^{Ab}	3.70±0.04^{Cb}	4.60±0.06^{Ab}

Values are mean ± S.E. Different upper case letters indicate significant difference between times within the same concentration and different lower case letters indicate significant difference between concentrations at a given sampling by two-way ANOVA and Tukey's test. No significant differences were observed in cell area, minor axis or major axis.

Table 3 Biochemical parameters in *Rhamdia quelen* subjected to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h.

Groups/ Parameters	cortisol (ng mL^{-1})	glucose (mg dL^{-1})	lactate (mg dL^{-1})	protein (g dL^{-1})	ammonia (mg L^{-1})	urea (mg dL^{-1})	AP (U L^{-1})	ALT (U mL^{-1})	AST (U mL^{-1})
0 mg L^{-1} propofol									
1 h	403.49 ± 48.51^{Aa}	34.48±3.88	17.07 ± 1.81^{Aa}	5.28±0.40	4.16±0.11	23.47±2.48	23.19 ± 1.45^{Aa}	22.70±1.96	23.51±1.72
6 h	248.07 ± 7.47^{Ba}	34.54±1.82	16.73 ± 1.55^{Aa}	4.73±0.27	4.32±0.23	22.33±3.13	21.59 ± 2.42^{Aa}	22.59±2.33	20.72±2.16
12 h	191.24 ± 19.68^{Ca}	36.27±3.67	13.22 ± 1.52^{Aa}	5.33±0.29	4.03±0.13	27.17±3.54	33.72 ± 2.60^{Aa}	17.11±2.03	23.14±2.43
0.4 mg L^{-1} propofol									
1 h	211.63 ± 38.50^{Ab}	46.67±4.67	12.17 ± 1.05^{Aab}	4.98±0.40	4.08±0.18	34.90±4.55	36.70 ± 3.95^{Aa}	26.05±1.53	25.22±1.29
6 h	241.68 ± 27.78^{Ba}	44.33±4.87	10.83 ± 1.38^{Aab}	5.28±0.26	3.80±0.15	27.83±3.36	28.47 ± 3.36^{Aa}	23.20±1.29	27.89±3.56
12 h	246.82 ± 32.32^{Bb}	48.71±1.27	10.66 ± 1.26^{Aa}	5.67±0.30	3.79±0.08	32.01±4.79	35.62 ± 3.65^{Aab}	21.23±2.42	26.92±2.54
0.8 mg L^{-1} propofol									
1 h	71.70 ± 15.35^{Ac}	48.03±3.81	7.32 ± 0.43^{Ab}	5.66±0.19	3.41±0.26	29.04±2.69	26.40 ± 2.39^{Aa}	27.80±1.43	28.28±2.46
6 h	166.38 ± 39.41^{Bb}	44.27±4.34	7.34 ± 0.79^{Ab}	5.15±0.17	3.85±0.31	18.01±1.60	20.73 ± 1.98^{Aa}	27.25±1.93	23.09±2.44
12 h	200.28 ± 18.84^{Ca}	38.13±2.32	9.18 ± 1.64^{Aa}	5.78±0.24	3.47±0.13	24.55±2.39	47.80 ± 4.70^{Bb}	21.01±1.48	26.91±2.11

Values are mean±S.E. AP=alkaline phosphatase, ALT=alanine aminotransferase and AST=aspartate aminotransferase. Different upper case letters indicate significant difference between times within the same concentration and different lower case letters indicate significant difference between concentrations at a given sampling time by two-way ANOVA and Tukey's test or the Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Nemenyi test. No significant differences were observed in glucose, protein, ammonia, urea, ALT or AST.

3.3 ARTIGO 3

Histological and antioxidant responses in *Rhamdia quelen* sedated with propofol

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Abstract

Morphometry of gills and antioxidant/oxidant status in gills, brain, liver and blood of *Rhamdia quelen* sedated with propofol were studied. The purpose was to investigate structural and functional responses upon administration of the drug in order to validate its use for the species. The fish were exposed to 0, 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h, which are times normally used in live fish transport. Propofol induced an increase in chloride cell in the non-respiratory epithelium of the gill. Standard biochemical assays (thiobarbituric acid reactive substances and lipid hydroperoxides) indicated that the lowest concentration of propofol did not induce lipoperoxidation in gills, brain, liver or blood. The oxidative status of enzymatic (catalase, superoxide dismutase and glutathione-S-transferase) and non-enzymatic (non-protein thiols) antioxidants differed among the tested sites. Apart from the blood, superoxide dismutase was the enzyme to show the highest activity in the presence of propofol, which is known to possess antioxidant properties that resemble those of vitamin E. The absence of chloride cells in the respiratory epithelium (lamella) in association with the stability of the lamellar structure (i.e. lamellar area, total height of lamella and width of lamella) indicate that ion and oxygen uptake were preserved under propofol sedation. Thus, 0.4 mg L^{-1} propofol should be considered to sedate *R. quelen* during lengthy procedures, such as transport, for it was able to maintain ionic and respiratory homeostasis as well as prevent peroxidative damage in vital organs.

Keywords: silver catfish, sedation, gill morphology, chloride cells, oxidative stress

Introduction

Live fish transport is likely to involve stress, trauma and the onset of post-transfer diseases especially due to deterioration of water parameters (Treasurer 2012). In order to avoid such potential problems, light sedation during the transportation process is beneficial for it reduces motion, ventilation and responsiveness to stimuli. The outcome is a state of calmness that helps preserving fish welfare as well as maintaining water quality, since there is a decline in oxygen consumption and in carbon dioxide and ammonia output (Ross & Ross 2008).

Determination of sedative concentrations and exposure times is crucial in order to guarantee the efficacy of the procedure. A given fish species at an specific stage of life will respond differently to such variables, hence the need to establish the most reliable protocol according to the biological features as well as the environmental characteristics (Ross & Ross 2008).

Propofol, a strong positive modulator of $GABA_A$ receptor, is widely used for intravenous sedation and anaesthesia of animals including fish (Fleming, Heard, Floyd & Riggs 2003). In fish, nonetheless, its use is also effective via immersion (Gressler, Parodi, Riffel, da Costa & Baldisserotto 2012a). A recent study has proven propofol as capable of blocking cortisol release and preserving the stability of some haematological, biochemical and hydromineral biomarkers of stress in propofol-bathed silver catfish (*Rhamdia quelen*) for prolonged periods (Gressler, Sutili, da Costa, Parodi, Pês, Koakoski, Barcellos & Baldisserotto 2014).

The gills have a complex internal organization that performs vital functions such as respiration, osmoregulation and excretion (Wendelaar-Bonga 1997; Cengiz 2006), thus the assessment of their structural and functional integrity is highly representative of the fish response towards the solubilized drug. Further, the gill epithelium is the main route of

sedative/anaesthetic entry and excretion, and some studies have described gill irritability as a side effect of such agents (Ross & Ross 2008). The aim of this study was to investigate the effects of propofol on gill morphometry and oxidative status in gills, brain, liver and blood of *R. quelen*. The present study provides the first insights into the effects of propofol on these parameters.

Materials and methods

Fish

Farmed juvenile *R. quelen* (n = 90; 91.44 ± 1.98 g, 20.66 ± 0.15 cm) were transferred to the Laboratório de Fisiologia de Peixes (LAFIPE) at Universidade Federal de Santa Maria (UFSM). The fish were acclimated for seven days in 250 L tanks (15 fish/tank) containing dechlorinated well water (200 L/tank) in a semi-static system with permanent aeration (21.5 ± 0.08 ºC; pH 7.45 ± 0.13; dissolved oxygen 8.04 ± 0.26 mg L^{-1}). Water changes were made every second day and fish were fed commercial feed pellets (42% extruded crude protein; 4% fibrous matter; 14% mineral matter; 2.5% calcium) once a day until satiation. The procedures were conducted with the approval of the Ethics Committee on Animal Experimentation of the UFSM (registration n° 67/2012).

Sedative

Propofol (Propotil 1%; BioChimico, BR; www.biochimico.com.br) was purchased and a pilot study was done based on previous results (Gressler *et al*. 2012a). Two concentrations of the drug, 0.4 and 0.8 mg L^{-1}, which induced up to stages 2 and 3a of sedation (Schoettger & Julin 1967), were chosen to be tested on the fish during periods which are normally used in live fish transport, 1, 6 and 12 h.

Experimental design

Food was withdrawn from the animals 24 h before testing. The fish were then exposed to 0 (control), 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h, totalling 9 groups (10 fish/group, two replicates of five fish each). The experiment was carried out in controlled laboratory conditions in order to avoid interferences such as decline in dissolved oxygen, build-up of ammonia, accumulation of carbon dioxide and reduction in pH, since the aim was to investigate the sole effect of the sedative exposure upon the assessed biomarkers. The water parameters were: 21.8 ± 0.12 ºC and dissolved oxygen 6.65±1.0 mg L^{-1} (Yellow Springs Instruments, Yellow Springs, OH, USA; model Y55), pH 7.4 ± 0.06 (Hanna Instruments, Woonsocket, RI, USA; model HI 8424), total ammonia 0.14 ± 0.02 mg L^{-1} (Verdouw, Van Echaematocriteld & Dekkers 1978) and un-ionized ammonia 0.002 ± 0.001 mg L^{-1} (Colt 2002). No fish died during the course of the experiment.

Exposure was made in 40 L tanks (static system) filled to 50% with the same water used during acclimation (20 L/tank). The fish were hand-transferred to the experimental tanks and maintained in the confined conditions for the appointed time. They were subsequently removed from the water for blood sampling (1 mL) via the caudal peduncle with heparinized sterile syringes and for biometrics. Euthanasia by transection of the spinal cord was performed and the gills, liver and brain were promptly dissected out for histological analyses (at all exposure times) and/or oxidative stress analyses (at 6 and 12 h).

Histological analyses

The third left gill arch was prepared for light microscopy. Subsequently to excision, it was rinsed in saline buffer and immersed in Bouin's solution for 24 h. Then its central portion was carefully excised, dehydrated in graded ethanol concentrations and embedded in Technovit 7100 resin. A sagittal cut was obtained on a LEICA RM2245 microtome set to 1-2 µm, mounted on a glass slide and stained with toluidine blue. Examination was performed at 400x using a Zeiss Axio Vision System with Remote Capture 4.7 Rel DC - Cannon Power shot G9.

The slides were thoroughly examined in order to determine the presence of histopathological alterations. In addition, six high-power fields were randomly selected on each slide to measure lamellar area, total height of lamella, height of potentially functional lamella, width of lamella, diffusion distance, interlamellar distance and filament epithelium thickness (modified from Hughes 1984). Chloride cells were counted in 1 mm of filament (Figure 1).

Prooxidants and antioxidants analyses in tissues

The remaining gill structure, the brain and the liver were frozen in liquid nitrogen as soon as they were excised. The tissues were kept at -80°C pending homogenization, which was carried out as outlined in Gressler, Riffel, Parodi, Saccol, Koakoski, da Costa, Pavanato, Heinzmann, Caron, Schmidt, Llesuy, Barcellos & Baldisserotto (2012b), and measurement of oxidative stress parameters in the supernatants.

Protein content was measured (Lowry, Rosebrough, Farr & Randall 1951) using serum albumin as a standard. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) assay (Buege & Aust 1978) and by determination of lipid hydroperoxides (LOOH) (Södergren, Nourooz-Zadeh, Berglund & Vessby 1998). Absorbance was determined in spectrophotometer at 535 and 560 nm, respectively, and the results are presented as nmol mg protein^{-1}.

Catalase (CAT) activity was assayed by measuring the decrease in the degradation of hydrogen peroxide at 240 nm (Boveris & Chance 1973) and the results are reported as pmol mg protein^{-1}. Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm (Misra & Fridovich 1972). The enzyme activity is expressed as SOD units mg protein^{-1}. To determine glutathione-S-transferase (GST) activity, the rate of dinitrophenyl-S-glutathione formation at 340 nm was evaluated (Habig, Pabst & Jakoby 1974). The results are presented as pmol min^{-1} mg protein^{-1}. Assessment of non-protein thiols (NPSH), which are non-enzymatic antioxidants and represent an indirect

measure of reduced glutathione (GSH), was based on GSH reacting with 5,5'-dithiobis-(2-nitrobenzoic acid). The addition of 0.5 M perchloric acid allowed for the elimination of proteins. The samples were read spectrophotometrically at 412 nm (Ellman 1959) and NPSH content is expressed as µmol mg protein^{-1}.

Prooxidants and antioxidants analyses in blood

An aliquot of the blood sampled at 6 and 12 h was destined for measurement of oxidative stress parameters. It was centrifuged in heparinized vials at 1110 xg for 5 min and the obtained red blood cells (RBC) were used to estimate lipid peroxidation by TBARS, as well as enzymatic and non-enzymatic antioxidants. In the case of TBARS, 4 mM magnesium sulfate and 1 mM acetic acid were added to the sample. For the antioxidants evaluation, 20% trichloroacetic acid (TCA) was added. The sample was spun at 1110 xg for 5 min and the supernatant was reserved to be assayed as previously described for the tissues.

Statistical analyses

All data were analysed by Statistica software 7.0 (Stat Soft. Inc., wwwstatsoft.com). Bartlett test was applied to assess normality and Levene test was used to verify homogeneity of variances. Two-way ANOVA and Tukey's test were used for parametric analyses and the non-parametric analyses were made with the Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Nemenyi test. Differences were considered significant at $P < 0.05$. Data are presented as the mean ± SD.

Results

Histological findings

Lamellar aneurysms were observed in some fish throughout all groups. Lamellar area, total height of lamella, height of potentially functional lamella, width of lamella, diffusion distance and filament epithelium thickness were not affected by the treatments. Significant

differences were observed in interlamellar distance at 6 and 12 h. Exposure to propofol increased the number of chloride cells in the interlamellar region of the filament (Table 1).

Prooxidants and antioxidants in tissues

Gills

At 12 h TBARS levels were 26% higher at 0.8 mg L^{-1} propofol than in control group, while at 6 h LOOH levels increased 34% at 0.8 mg L^{-1} propofol compared to control. SOD activity rose 42% in the presence of propofol at 6 h and 28% at 0.8 mg L^{-1} propofol in relation to the other concentrations at 12 h. At 6 h GST activity was 28% higher at 0.8 mg L^{-1} propofol than control; at 0.4 mg L^{-1} propofol the enzyme activity was 25% lower at 12 h compared to 6 h (Table 2).

Brain

The activity of CAT declined 38% at 0.8 mg L^{-1} propofol in the fish sampled at 12 h compared to control. At 6 h SOD activity was 23% higher at 0.4 mg L^{-1} propofol than control, and it was 12% higher at 0.8 mg L^{-1} propofol than at the intermediary concentration; the enzyme activity was also 37% higher at 0.8 mg L^{-1} propofol than at the remaining concentrations at 12 h (Table 2).

Liver

At 6 h the activity of SOD increased 104% upon exposure to propofol (Table 2).

Prooxidants and antioxidants in blood

The contents of TBARS in RBC at 0.8 mg L^{-1} propofol augmented 80% at 12 h comparing to 6 h as well as to 12 h in control group. At 6 h GST activity increased 55% at 0.8 mg L^{-1} compared to control. At 12 h NPSH contents were 37% lower at 0.4 mg L^{-1} propofol

than at the remaining concentrations. In the fish exposed to 0.8 mg L^{-1} propofol NPSH levels were 94% higher at 12 h than at 6 h (Table 2).

Discussion

Fish gills act as an interface between the organism and the aquatic environment, thus being the primary uptake site for substances dissolved in water (Cengiz 2006). Histopathological gill analyses have shown detrimental effects caused by organic toxicants, such as haemorrhages with rupture of epithelium, epithelial hyperplasia and fusion of adjacent lamella (Sensini, Torre, Corsi & Focardi 2008). Extensive gill changes may impair physiological function, rendering the fish unable to osmoregulate, ventilate and maintain acid-base balance (Wendelaar-Bonga 1997). In the current study, a few lamellar aneurysms were seen in some of the control and exposed fish; according to Posner, Scott & Law (2013), aneurysms usually arise from handling and euthanasia. Thus, it may be suggested that the sedative protocols tested on *R. quelen* did not promote the development of gill histopathologies. These findings are congruent with the macroscopic analyses, which had shown that the integrity of the gill structure and its proper reddish colour were preserved. Further, there was no presence of mucus, which proliferates as a means of protection or to facilitate ion regulation when fish are facing undesirable conditions (Tkatcheva, Hyvärinen, Kukkonen, Ryzhkov & Holopainen 2004).

The morphometric evaluation of the gill structure evidenced only minor adjustments in interlamellar distance. It may be inferred that the range observed for this index is normal for this fish species, which is confirmed by the absence of variation in parameters as width of lamellae and diffusion distance. Bindon, Gilmour, Fenwick & Perry (1994) and Greco, Fenwick & Perry (1996), in turn, reported greater diffusion distance and reduced interlamellar distance as a result of epithelium thickening caused by lamellar chloride cell proliferation.

Although chloride cells proliferated in the presence of propofol, they were confined to the filament epithelium. These cells are also called ionocytes for being specialized in ionic regulation, and are typically present at the trailing edge of the filament. They might inundate the lamellae when proliferation occurs, as a result of exposure to soft water (Greco *et al.* 1996) or toxicants (Tkatcheva *et al.* 2004; Sensini *et al.* 2008), for example. Once in the lamella, the chloride cells are closer to the bloodstream, which helps sustaining ion uptake. Nonetheless, it is likely to impair gas transfer (O_2 uptake and CO_2 excretion), since thickening of the lamellar epithelium increases blood-to-water diffusion distance (Bindon *et al.*1994; Greco *et al.* 1996). The increase in chloride cell number in the filament epithelium in the current investigation may have been an adaptive response to optimize gill ion transport in the altered milieu, but the extent of proliferation was not great enough to induce filament epithelium thickness.

When increases in gill area are elicited in order to absorb more O_2, freshwater fish experience increased water influx and ion losses (Wendelaar-Bonga 1997). A certain degree of reduced oxygenation should be expected during sedation/anaesthesia, but since remodelling of the respiratory epithelium was not observed in sedated *R. quelen*, it may be suggested that the level of respiratory depression imposed by propofol was not great enough to limit oxygen absorption. Nonetheless, insufficient gill ventilation, which may lead to severe hypoxia and respiratory and metabolic acidosis, has been commonly described in sedated/anesthetized fish (Ross & Ross 2008).

Propofol has been proven to increase the antioxidant capacity of tissues and RBC, thus decreasing reactive oxygen species (ROS)-induced lipid peroxidation, the major contributor to the loss of cell function (Runzer, Ansley, Godin & Chambers 2002; Feng, Bai, Ma & Wang 2008). The antioxidant effect of propofol arises from the ability of its phenolic structure, which is similar to that of the endogenous antioxidant α-tocopherol (vitamin E), to react with

free radicals and form a phenoxyl radical, a relatively stable intermediate (Green, Bennet & Nelson 1994).

It is well-accepted that tissue reperfusion after transient oxygen deprivation results in oxidative stress, which may contribute to pathological injury of cells (Feng *et al*. 2008). Indeed, Gressler *et al*. (2012b) demonstrated that recovery from anaesthesia (reoxygenation) with MS-222 induced lipid peroxidation in liver of *R. quelen* when the antioxidant system was poorly triggered under the anaesthetic-induced hypoxia (± 3 min). Instead, when the antioxidant defences are fully enhanced during hypoxia, the 'preparation to oxidative stress' concept is clearly observed: the antioxidant capacity is built-up in anticipation of the ROS-induced damage associated to the hypoxic-normoxic transition (Hermes-Lima & Zenteno-Savín 2002). Nevertheless, Lushchak, Bagnyukova, Lushchak, Storey & Storey (2005) observed a rise in lipid peroxidation in common carp (*Cyprinus carpio*) liver in the course of prolonged hypoxia (5 h). Although there is a reduction in aerobic respiration, a decline in oxygen levels is bound to increase ROS production for both aerobic and anaerobic pathways generate reduced oxygen intermediates (Chandel, McClintock, Feliciano, Wood, Melendez, Rodriguez & Schumacker 2000). Accordingly, in gills of *R. quelen* exposed to 0.8 mg L^{-1} propofol, lipid peroxidation was characterized by increased levels of LOOH (6 h) and TBARS (12 h). De La Cruz, Sedeno, Carmona & Sanchez de la Cuesta (1998) proposed that propofol may act on the glutathione system and induce changes such as increased GST activity. In gills of propofol-sedated *R. quelen*, however, the rise observed in GST as well as in SOD was not prominent enough in order to neutralize the impact of peroxides. Green *et al*. (1994) suggested that propofol, which is a highly lipid soluble compound, accumulates in lipid fractions and performs its antioxidant radical scavenging effect in these compartments more prominently than within those aqueous ones.

In another study evaluating the redox status in gills of *R. quelen* transported under sedation with essential oil of *Lippia alba* for 5, 6 or 7 h (representing hyperoxia, normoxia and hypoxia, respectively), no changes were observed in SOD activity throughout the groups (Azambuja, Mattiazzi, Riffel, Finamor, Garcia, Heldwein, Heinzmann, Baldisserotto, Pavanato & Llesuy 2011). On the other hand, when Cooper, Clough, Farwell & West (2002) tested different oxygen tensions in spot (*Leiostomus xanthurus*), gill SOD activity increased as oxygen concentration declined. Víg & Nemcsók (1989) also reported that the oxygen-limited state significantly increased SOD activity in *C. carpio* gill. According to Cooper *et al*. (2002) and Hermes-Lima & Zenteno-Savín (2002), increased SOD activity is likely to be an important mechanism in order to break down ROS that arise on reoxygenation.

No lipid peroxidation was detected in *R. quelen* brain, which is a highly lipophilic environment. Moreover, SOD activity was enhanced in this organ in the presence of propofol. Azambuja *et al*. (2011), however, detected lipid peroxidation in brain of sedated *R. quelen* with no induction of antioxidant defences.

As well as the gills and the brain, *R. quelen* liver showed increased SOD activity under sedation. Víg & Nemcsók (1989) similarly found hepatic SOD activity to be increased in response to hypoxia exposure in *C. carpio*. Lushchak *et al*. (2005) cited that antioxidant defences are typically exacerbated in liver due to its key role in detoxification. Wilhelm Filho, Torres, Zaniboni-Filho & Pedros (2005) found liver TBARS contents of piapara (*Leporinus elongatus*) to be as high under moderate hypoxia exposure (7 days) as on return to normoxia, with higher values of antioxidant defences in the former. Lushchak, Lushchak, Mota & Hermes-Lima (2001), in turn, observed elevated CAT activity in goldfish (*Carassius auratus*) liver after 8 h of oxygen deprivation. The hepatic activity of CAT, which remained unchanged in propofol-sedated *R. quelen*, was the highest when compared to the other organs, corroborating previous findings (Cooper *et al*. 2002; Azambuja *et al*. 2011). *R. quelen* liver

also presented high levels of glutathione in its reduced form, GSH (measured through NPSH). According to Burnette, McCormick & Lewis (2013), glutathione is the main endogenous antioxidant produced by cells. Although the liver is a lipid-rich organ, thus prone to lipid peroxidation, maintenance of high constitutive levels of CAT and NPSH and up-regulation of SOD in the presence of the lipophilic sedative propofol were able to prevent oxidative stress in the hepatic tissue of *R. quelen*.

Red blood cells are permanently exposed to ROS due to their role in oxygen transport. In addition, the nucleated RBC of teleosts have an extra source of ROS because of the high rates of aerobic metabolism in their mitochondria (Burnette *et al*. 2013). At the highest concentration of propofol, the slight rise of red blood cell TBARS content may have resulted from the low enzymatic activities of SOD and GST. Wdzieczak, Zalesna, Wujec & Peres (1982) showed that fish RBC are highly protected against oxygen damage, but that was restricted to CAT and NPSH in this study. Blood catalase activity was one of the highest among the assessed endogenous sites of enzymatic production, only second to the liver. As for the non-enzymatic antioxidant, high levels of glutathione have been described in fish RBC with the aim of protecting the haemoglobin, which has a higher propensity to oxidation in comparison to that of other vertebrates, from spontaneous oxidation to metahaemoglobin (Martínez-Álvarez, Morales & Sanz 2005).

Histological and antioxidant responses of *R. quelen* exposed to propofol suggest that the lowest concentration of the sedative tested was able to maintain ionic and respiratory homeostasis as well as enhance antioxidant activity and prevent oxidative damage in vital organs. With regard to the antioxidant defences, up-regulation of SOD, a primary antioxidant enzyme, was detected in all of the analysed organs in the presence of propofol, which is known for its antioxidant properties. The fact that a drug may boost the body defences while sedating seems to be of a great value, since peroxidative injury is expected to rise when

sedation ceases. Thus, propofol at 0.4 mg L^{-1} has the potential for being used as a sedative for *R. quelen* during extended procedures such as transport.

Acknowledgements

Funding for this research was provided by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul/Programa de Apoio a Núcleos de Excelência (FAPERGS/PRONEX), Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Tecnológico (CNPq). Authors would like to thank CNPq for providing research fellowship for Bernardo Baldisserotto and CAPES and FAPERGS for graduate fellowships to Luciane Tourem Gressler, Fernando Jonas Sutili, Etiane Medianeira Saccol, Tanise Silva Pês and Thaylise Vey Parodi. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Figure legend

Figure 1. Gill histology parameters analyzed in *Rhamdia quelen* through light microscopy. Black letters: A=lamellar area; B=total height of lamella; C=height of potentially functional lamella; D=width of lamella; E=diffusion distance; F=interlamellar distance; G=filament epithelium thickness (modified from Hughes, 1984). White letters: F=filament; L=lamella. Arrow indicates chloride cell. Scale bar: 20 µm.

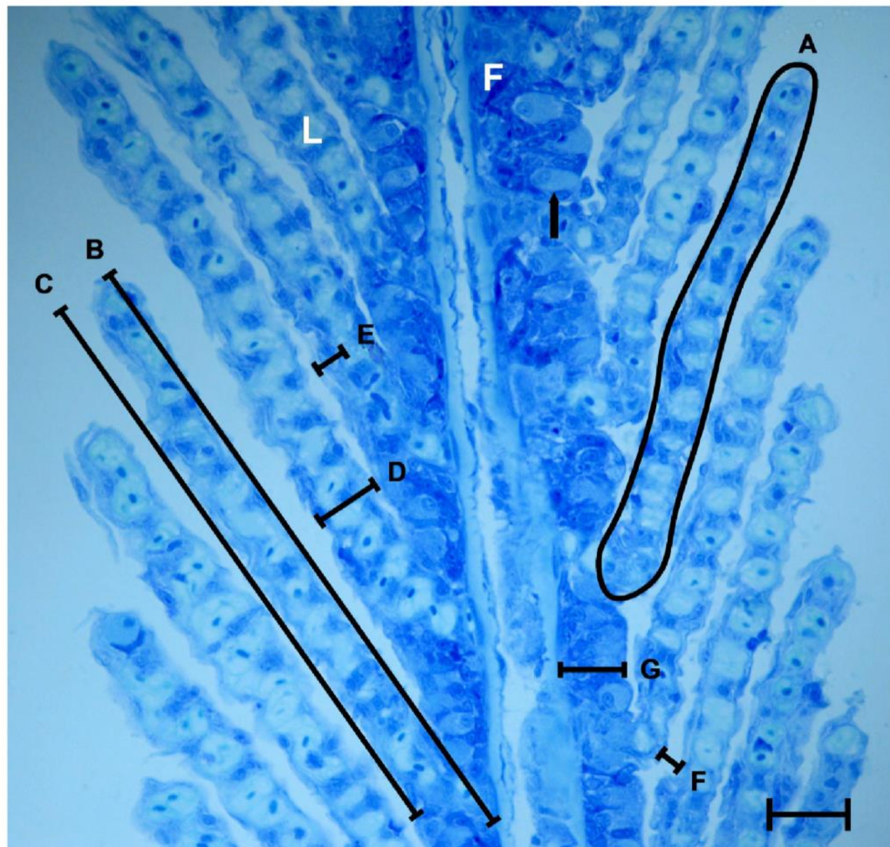

Figure 1

Table 1. Gill histology parameters in *Rhamdia quelen* subjected to 0 (control), 0.4 or 0.8 mg L^{-1} propofol for 1, 6 or 12 h.

	Lamellar area	Total height of lamella	Height of potentially functional lamella	Width of lamella	Diffusion distance	Interlamellar distance	Filament epithelium thickness	Chloride cells
0 mg L^{-1}								
1 h	4470.36±977.25	130.29±35.24	103.43±29.17	28.88±5.42	9.02±1.45	$28.68±3.62^{ABa}$	37.11±10.82	$23.45±3.53^{Aa}$
6 h	4877.98±1352.17	142.65±38.72	107.21±32.97	36.03±6.02	8.75±1.20	$35.04±5.02^{Aa}$	39.06±7.64	$21.08±2.44^{Aa}$
12 h	5657.60±1210.20	144.03±29.60	108.98±26.18	31.17±6.06	8.67±1.70	$25.10±4.64^{Ba}$	39.93±10.86	$19.75±2.13^{Aa}$
0.4 mg L^{-1}								
1 h	6088.43±1378.62	133.65±45.54	102.13±34.12	34.09±5.28	10.16±1.13	$26.04±3.81^{Aa}$	41.15±9.66	$31.58±4.85^{Ab}$
6 h	5559.03±1093.27	153.21±30.91	113.65±32.62	30.55±3.74	9.03±0.69	$27.42±5.84^{Ab}$	36.88±7.05	$38.72±1.81^{Bb}$
12 h	5934.22±1773.88	155.10±39.10	115.06±37.33	33.81±7.54	9.75±1.30	$29.81±5.50^{Aab}$	48.79±13.06	$42.03±1.83^{Cb}$
0.8 mg L^{-1}								
1 h	5034.49±1163.68	140.57±28.14	107.20±23.28	32.79±5.81	9.90±0.85	$30.50±3.80^{Aa}$	43.59±12.19	$42.19±4.30^{Ac}$
6 h	6204.61±1310.71	157.87±32.68	212.16±29.92	35.82±6.64	10.19±1.15	$30.25±5.10^{Aab}$	47.53±7.27	$44.92±4.56^{Ac}$
12 h	5929.47±1191.83	166.11±19.55	131.77±19.65	33.68±4.87	9.67±1.33	$33.98±6.80^{Ab}$	44.32±8.95	$46.03±2.23^{Ac}$

Lamellar area in μm^2 and chloride cells in number mm^{-1}; the remaining parameters in µm. Values are mean ± SD. Different uppercase letters indicate significant difference between times in the respective concentration, and different lowercase letters indicate significant difference between concentrations in the respective time (symbols only shown where difference exists, $p < 0.05$).

Table 2. Oxidative parameters in gills, brain, liver and blood of *Rhamdia quelen* subjected to 0 (control), 0.4 or 0.8 mg L^{-1} propofol for 6 or 12 h.

TBARS=thiobarbituric acid reactive substances; LOOH=lipid hydroperoxides;

	TBARS nmol mg protein $^{-1}$	**LOOH** nmol mg protein^{-1}	**CAT** pmol mg protein^{-1}	**SOD** units mg protein^{-1}	**GST** pmol min^{-1} mg protein^{-1}	**NPSH** µmol mg protein^{-1}
GILLS						
0 mg L^{-1}						
6 h	$0.37{\pm}0.05^{a}$	$10.05{\pm}1.70^{a}$	0.39±0.06	$0.36{\pm}0.11^{a}$	$1.28{\pm}0.20^{a}$	--
12 h	$0.36{\pm}0.06^{a}$	$11.58{\pm}1.50^{a}$	0.32±0.05	$0.50{\pm}0.23^{a}$	$1.42{\pm}0.11^{ab}$	--
0.4 mg L^{-1}						
6 h	$0.42{\pm}0.11^{a}$	$11.78{\pm}2.33^{ab}$	0.35±0.05	$0.62{\pm}0.23^{b}$	$1.51{\pm}0.19^{ab}$	--
12 h	$0.42{\pm}0.09^{ab}$	$12.35{\pm}1.59^{a}$	0.37±0.06	$0.50{\pm}0.19^{a}$	$1.21{\pm}0.22^{a*}$	--
0.8 mg L^{-1}						
6 h	$0.41{\pm}0.06^{a}$	$13.51{\pm}3.16^{b}$	0.38±0.09	$0.61{\pm}0.13^{b}$	$1.64{\pm}0.16^{b}$	--
12 h	$0.47{\pm}0.08^{b}$	$13.83{\pm}2.02^{a}$	0.37±0.07	$0.76{\pm}0.18^{b}$	$1.50{\pm}0.23^{b}$	--
BRAIN						
0 mg L^{-1}						
6 h	1.23±0.24	12.11±0.91	$0.30{\pm}0.06^{a}$	$2.23{\pm}0.77^{a}$	1.02±0.31	--
12 h	1.34±0.35	13.00±0.81	$0.42{\pm}0.07^{a}$	$2.12{\pm}0.26^{a}$	1.05±0.18	--
0.4 mg L^{-1}						
6 h	1.45±0.16	12.97±1.13	$0.40{\pm}0.10^{a}$	$2.74{\pm}0.23^{b}$	1.07±0.17	--
12 h	1.45±0.35	12.71±1.04	$0.37{\pm}0.12^{ab}$	$2.21{\pm}0.44^{a*}$	0.99±0.25	--
0.8 mg L^{-1}						
6 h	1.09±0.28	13.16±1.21	$0.30{\pm}0.10^{a}$	$3.07{\pm}0.33^{c}$	0.98±0.26	--
12 h	1.24±0.16	12.69±1.24	$0.26{\pm}0.07^{b}$	$3.02{\pm}0.53^{b}$	1.03±0.21	--
LIVER						
0 mg L^{-1}						
6 h	0.56±0.16	13.04±1.79	2.87±0.53	$0.78{\pm}0.15^{a}$	1.16±0.57	17.76±3.60
12 h	0.51±0.23	14.74±3.63	3.05±0.97	$1.56{\pm}0.43^{a}$	1.12±0.50	19.68±4.51
0.4 mg L^{-1}						
6 h	0.50±0.12	13.11±2.27	2.89±0.65	$1.59{\pm}0.38^{b}$	1.25±0.67	16.46±3.46
12 h	0.49±0.37	11.49±2.93	2.60±0.64	$1.42{\pm}0.39^{a}$	1.05±0.35	15.53±3.61
0.8 mg L^{-1}						
6 h	0.42±0.17	13.18±1.33	2.86±0.56	$1.58{\pm}0.51^{b}$	1.01±0.25	14.35±4.23
12 h	0.52±0.23	11.74±1.96	2.56±0.57	$1.30{\pm}0.42^{a}$	1.08±0.47	15.67±5.70
RBC						
0 mg L^{-1}						
6 h	$0.06{\pm}0.01^{a}$	--	0.61±0.05	0.36±0.10	$0.22{\pm}0.07^{a}$	$13.09{\pm}2.01^{a}$
12 h	$0.05{\pm}0.01^{a}$	--	0.63±0.12	0.30±0.09	$0.23{\pm}0.05^{a}$	$15.73{\pm}3.54^{a}$
0.4 mg L^{-1}						
6 h	$0.05{\pm}0.01^{a}$	--	0.61±0.12	0.36±0.06	$0.26{\pm}0.06^{ab}$	$12.73{\pm}3.49^{a}$
12 h	$0.05{\pm}0.01^{a}$	--	0.64±0.07	0.30±0.09	$0.33{\pm}0.11^{a}$	$9.93{\pm}1.80^{b}$
0.8 mg L^{-1}						
6 h	$0.05{\pm}0.01^{a}$	--	0.57±0.11	0.24±0.07	$0.34{\pm}0.06^{b}$	$7.61{\pm}1.42^{b}$
12 h	$0.09{\pm}0.01^{b*}$	--	0.54±0.09	0.29±0.09	$0.32{\pm}0.11^{a}$	$14.78{\pm}3.84^{a*}$

CAT=catalase; SOD=superoxide dismutase; GST=glutathione-S-transferase; NPSH=non-protein thiols; RBC=red blood cells. Values are mean ± SD. Different lowercase letters indicate significant difference between concentrations in the respective time, and (*) indicates significant difference from 6 h in the respective concentration (symbols only shown where difference exists, $p < 0.05$). (--) indicates parameter not measured.

4 DISCUSSÃO

Este estudo avaliou o potencial de três anestésicos com características distintas para uso na sedação e/ou anestesia de peixes: MS222, OE de *A. triphylla* e propofol. O MS222 é o clássico anestésico para peixes (CARTER et al., 2011; READMAN et al., 2013; TRUSHENSKI et al., 2013). Há estudos empregando este fármaco em muitas espécies para avaliação de parâmetros não relacionados à atividade anestésica, justamente pelo seu uso difundido (MURRAY, 2002; HERNANDEZ-DIVERS et al., 2004; NEIFFER, STAMPER, 2009). Já o OE *A. triphylla* foi recentemente patenteado como anestésico para espécies aquáticas (HEINZMANN et al., 2011), dentre elas os peixes, e são poucos os estudos apresentando resultados relacionados a esta aplicação (PARODI et al., 2012, 2014). E o propofol, amplamente utilizado nas medicinas humana e veterinária devido a sua potente ação anestésica central, sendo tipicamente administrado via endovenosa (ANDREWS et al., 1997; CARPENTER, 2005; GARCIA, KOLESKY, JENKINS, 2010). Seu uso na anestesia de peixes em banho de imersão iniciou apenas recentemente, havendo um limitado número de relatos a cerca do seu desempenho para este fim (PEYGHAN et al., 2008; GHOLIPOURKANANI, AHADIZADEH, 2013; GOMULKA et al., 2014; VALENÇA-SILVA et al., 2014; OSTRENSKY, PEDRAZZANI, VICENTE, 2015).

O MS222 tem sido utilizado como o anestésico de primeira escolha para peixes em diversos países há muitos anos (FDA, 2006; HEALTH CANADA, 2011; VETERINARY MEDICINES DIRECTORATE, 2011). No entanto, não está comercialmente disponível em regiões como a América do Sul, por exemplo. Assim, deve-se importá-lo a um custo que inviabiliza a aplicabilidade na piscicultura brasileira (ROUBACH, GOMES, VAL, 2001). Além disso, os resultados obtidos em jundiás no presente estudo apontaram sua ineficácia em manter a estabilidade de parâmetros fisiológicos durante o procedimento anestésico, permitindo a ativação do eixo HHI e a liberação de cortisol, por exemplo. Também foi observada a indução de lipoperoxidação na sua presença, indicando que não possui potencial antioxidante ou ainda que atua como agente oxidante. Sendo assim, seu uso no Brasil não se justifica, principalmente porque existem alternativas que podem ser obtidas no país e que desempenham um papel mais eficaz no contexto da anestesia de peixes, como o OE *A. triphylla* e o propofol.

O OE *A. triphylla* demonstrou um alto poder antioxidante em jundiá na presente investigação, provavelmente pela ação conjunta das enzimas CAT e GST hepáticas. Um dos constituintes majoritários do OE, o citral, pode ter exercido papel fundamental na indução da

GST. Além desta ação, o óleo possibilitou a depressão do sistema nervoso central, limitando a ativação do eixo HHI e a estabilidade nos níveis circulantes de cortisol. Ambas as ações não foram observadas nos peixes expostos ao MS222. Tais vantagens do óleo essencial foram evidenciadas apesar dos tempos de indução à anestesia terem sido no mínimo o dobro daquele sugerido na literatura (3 min) como ideal para evitar alterações fisiológicas, o que desmistifica a teoria de que um bom anestésico é aquele que promove indução em tempos curtos (GILDERHUS, MARKING, 1987). No entanto, aspectos referentes à sua farmacocinética e farmacodinâmica ainda devem ser elucidados e sua produção deve ocorrer em larga escala para que então possa ser estabelecido como anestésico para peixes. Mas o seu potencial foi novamente demonstrado neste estudo, somando aos resultados promissores já descritos anteriormente (PARODI et al., 2012, 2104) e assim auxiliando na compilação de informações sobre mais um importante papel deste produto originário de uma espécie de planta nativa, a qual é tão explorada na medicina popular (VALENTÃO et al., 2002; ZAMORANO-PONCE et al., 2006).

Diferentemente do OE *A. triphylla*, o propofol é comercializado mundialmente, com o benefício adicional de apresentar um preço acessível. O único entrave é a exigência de prescrição Médico Veterinária para uso em animais por ser medicamento controlado, mas que por outro lado evita seu uso indevido. O produto é obtido na forma aquosa, a qual possui grande hidrossolubilidade e assim proporciona fácil diluição na água (HIPPALGAONKAR, MAJUMDAR, KANSARA, 2010; MEYER, FISH, 2011). Os resultados observados em jundiá neste estudo demonstraram que mesmo concentrações muito baixas do fármaco promovem uma potente ação antioxidante, evidenciada especialmente pela indução da enzima SOD em todos os órgãos investigados (brânquias, encéfalo e fígado). Os órgãos lipofílicos, encéfalo e fígado, foram os locais onde houve maior atividade antioxidante, não ocorrendo lipoperoxidação em nenhuma das concentrações testadas. Além da ausência de estresse oxidativo, o propofol também permitiu a estabilidade estrutural e funcional do aparelho branquial mesmo após 12 h de exposição. Isto demonstra que o fármaco não interferiu em funções vitais como respiração e ionorregulação e não apresentou toxicidade, o que poderia ter induzido proliferação de muco e mudança na coloração, além de alterações estruturais e funcionais no tecido. Neste relato e em trabalhos prévios pôde-se verificar que o propofol explicita claramente a passagem de um estágio anestésico para o outro, o que implica em maior segurança durante o procedimento por evitar que a indução anestésica ocorra de maneira descontrolada e o animal aprofunde o nível de depressão central a ponto de este tornar-se irreversível (GRESSLER et al., 2012).

Diante dos achados reportados neste estudo, sugere-se que o OE *A. triphylla* continue sendo alvo de pesquisa para ampliar o conhecimento sobre aspectos como seu mecanismo de ação e efeitos farmacológicos, por exemplo. Tais investigações poderão embasar a decisão de produzi-lo em larga escala e assim ser validado como produto natural para uso na anestesia de peixes (inclusive na piscicultura orgânica), como ocorre com o eugenol, por exemplo, que é facilmente obtido no mercado local. Também sugere-se que o propofol seja continuamente estudado como anestésico para peixes em banho de imersão, visto que as vantagens já observadas indicam sua grande eficácia anestésica neste animais, assim como reportado extensamente em mamíferos, por exemplo. A intenção principal desta linha de pesquisa é explorar alternativas locais para uso na anestesia de peixes cultivados na piscicultura brasileira, em detrimento daquelas que apresentam um alto custo e dificuldade de obtenção, como o MS222. Os produtores, muitas vezes, dispensam o uso de anestésicos em procedimentos estressantes durante o manejo por causa do elevado custo. Assim, é de fundamental importância que a comunidade acadêmica proporcione aos piscicultores alternativas que possam favorecer a lucratividade da produção, sendo o bem-estar animal a premissa sustentadora desta importante relação pesquisa-extensão.

5 CONCLUSÃO

Este estudo apresentou dados que contribuem de maneira significativa para a área de Farmacologia Aplicada à Produção Animal, uma vez que demonstrou o potencial anestésico de alternativas farmacológicas para uso na sedação e anestesia de peixes. Através de ensaios hematológicos, bioquímicos, morfológicos, de estresse oxidativo e de indução e recuperação anestésica, comprovou-se que os produtos em avaliação, propofol e OE *A. triphylla*, produzem efeitos benéficos que se sobrepõe àqueles do anestésico utilizado internacionalmente para peixes, o MS222. Sendo assim, tanto o produto sintético quanto o natural podem ser explorados na piscicultura local, regional e nacional para obter os resultados desejáveis da sedação e da anestesia, preservando o bem-estar animal.

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