UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

AVALIAÇÃO DA EFICÁCIA DE NOVOS EXTRATOS VEGETAIS ANESTÉSICOS E DA TRICAÍNA PARA JUVENIS DE TAMBAQUI *Colossoma macropomum* (Cuvier, 1818): IMPLICAÇÕES SOBRE AS RESPOSTAS DE ESTRESSE

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Luis André Luz Barbas

Tese apresentada ao Programa de Pós-graduação em Aquicultura da Universidade Federal do Rio Grande - FURG, como requisito parcial à obtenção do título de Doutor.

Orientador: Prof. Dr. Luís André Nassr de Sampaio (FURG)
Coorientador: Prof. Dr. Luciano de Oliveira Garcia (FURG)

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DE DEFESA DA 34ª TESE DE DOUTORADO EM AQUICULTURA

No dia vinte e seis de agosto de dois mil e quinze, às oito horas e trinta minutos, no Auditório da Estação Marinha de Aquacultura da FURG, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, do Veterinário **LUIS ANDRÉ LUZ BARBAS**, orientado pelo Professor. Dr Luis André Nassr de Sampaio, composta pelos seguintes membros: Prof. Dr Luis André Nassr de Sampaio (Orientador – IO/FURG), Prof. Dr Luciano de Oliveira Garcia (Co-Orientador - IO/FURG), Prof. Dr. Ricardo Vieira Rodrigues (IO/FURG), Prof. Dr Ricardo Berteaux Robaldo (UFPel), Prof. Dr Sérgio Piedras (UFPel) e o Prof. Dr. Bernardo Baldisserotto (UFSM). Título da Tese: **"AVALIAÇÃO DA EFICÁCIA DE NOVOS EXTRATOS VEGETAIS ANESTÉSICOS E DA TRICAÍNA PARA JUVENIS DE TAMBAQUI *Colossoma macropomum* (Cuvier, 1818): IMPLICAÇÕES SOBRE AS RESPOSTAS DE ESTRESSE"**. Dando início à defesa, o Coordenador do PPGAq, Prof. Dr Marcelo Borges Tesser passou a presidência da sessão ao Prof. Dr. Luis André Nassr de Sampaio, que na qualidade de orientador, passou a palavra para o candidato apresentar a Tese. Após ampla discussão entre os membros da Banca e o candidato, a Banca se reuniu sob a presidência do Coordenador. Durante esse encontro ficou estabelecido que as sugestões dos membros da Banca Examinadora devem ser incorporadas na versão final, ficando a cargo do Orientador o cumprimento desta decisão. O candidato **LUIS ANDRÉ LUZ BARBAS** foi considerado **APROVADO**, devendo a versão definitiva da Tese ser entregue na Secretaria do PPGAq, no prazo estabelecido nas Normas Complementares do Programa. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pelo candidato e pelo Coordenador do PPGAq.

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Dedico

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RESUMO GERAL

Este trabalho teve por objetivo avaliar a eficácia do óleo essencial (OE) de *Nectandra grandiflora* Nees, do extrato de *Spilanthes acmella* var *oleracea* obtido por técnica de extração por fluido supercrítico com uso de CO_2 e da tricaína (MS-222) como anestésicos para juvenis de tambaqui *Colossoma macropomum* (Cuvier, 1818), além de seus efeitos sobre os parâmetros fisiológicos e de estresse oxidativo nos períodos pós-anestesia e pós-transporte sob sedação. Os peixes (3,3 a 65,74 g) foram aclimatados por 15 dias em sistemas de recirculação e os parâmetros de qualidade de água foram mantidos dentro da faixa de conforto para a espécie. Pelo menos cinco concentrações de extrato de *S. acmella*, OE de *N. grandiflora* e tricaína foram testados e as faixas-teste de concentrações foram determinadas através de ensaios preliminares. A concentração de 20 mg L^{-1} de extrato de *S. acmella* foi necessária para promover anestesia rápida (tempo de indução < 3 min) e profunda de juvenis de tambaqui (46,6 g) que também foram avaliados no período pós-anestesia quanto às respostas secundárias de estresse durante 72h na recuperação. Alterações fisiológicas transitórias ocorreram principalmente entre 2 até 24 h pós-anestesia com extrato de *S. acmella*, sendo que todas as variáveis de fisiologia retornaram ao normal ao final de 72 h na recuperação, à exceção das concentrações mais baixas de Na^+ sanguíneo que persistiram reduzidas em comparação aos valores de referência. A tricaína mostrou-se eficiente para anestesia rápida e profunda com concentrações a partir de 240 mg L^{-1} e não foram observadas vantagens claras de seu uso no transporte por até 10 h na concentração de sedação de 20 mg L^{-1} para atenuação de estresse oxidativo. O OE de *N. grandiflora* necessitou de concentração de 200 µL L^{-1} para determinar anestesia profunda, entretanto, não determinou anestesia rápida. O extrato de *S. acmella* na concentração de 10 mg L^{-1} foi suficiente para promover anestesia rápida e profunda dos juvenis (3,3 g) submetidos aos banhos anestésicos. Após o transporte por 2, 6 e 10 h na presença ou ausência do OE de *N. grandiflora* e extrato de *S. acmella* (30 µL L^{-1} e 1 mg L^{-1}, respectivamente) tecidos dos juvenis (músculo, fígado, cérebro e brânquias) foram avaliados quanto às respostas de estresse oxidativo através dos seguintes indicadores: capacidade antioxidante total (ACAP), atividade de GST e substâncias reativas ao ácido tiobarbitúrico (TBARS). Essas concentrações de sedação de OE de *N. grandiflora* e de de extrato de *S. acmella* foram eficazes para o transporte dessa espécie uma vez que proporcionaram proteção contra dano oxidativo principalmente nos músculos e brânquias. Juvenis transportados por 2, 6 e 10 h na presença de extrato de *S. acmella* (1 mg L^{-1}) e de tricaína (20 mg L^{-1}) não apresentaram diferenças significativas em relação aos animais transportados sem anestésicos quanto às respostas secundárias de estresse no sangue, ao passo que o OE de *N. grandiflora* na concentração de 30 mg L^{-1} atenuou essas respostas principalmente pela diminuição ou manutenção dos níveis glicêmicos e manutenção das concentrações normais de Na^+ no sangue. Todos os anestésicos utilizados nesse estudo foram eficazes e seguros para promover anestesia profunda com recuperação plena em juvenis de tambaqui. Os extratos das plantas *S. acmella* e *N. grandiflora* são recomendáveis para o transporte desta espécie em sacos plásticos por até 10 h, uma vez que houve relativa melhora das respostas secundárias de estresse e o processo de lipoperoxidação nos tecidos foi reduzido na presença dos extratos.

Palavras–chave: Anestesia, estresse oxidativo, transporte, MS-222, *Spilanthes acmella*, *Nectandra grandiflora.*

ABSTRACT

This study aimed to evaluate the efficacy of the essential oil (EO) of *Nectandra grandiflora* Nees, extract of *Spilanthes acmella* var *oleracea* obtained through supercritical fluid extraction methodology with the use of CO_2 and tricaine (MS-222) as anaesthetics for juvenile tambaqui *Colossoma macropomum* (Cuvier, 1818), as well as their impact on physiological balance and oxidative stress after deep anaesthesia and transport with sedation. Fish (3.3 to 65.74 g) were acclimated for 15 days in recirculation systems with daily partial changes of water. Water quality parameters were controlled and adequately maintained for the species. At least five concentrations of *S. acmella* extract, EO of *N. grandiflora* and tricaine were tested and concentrations ranges were determined in preliminary tests. The dosage of 20 mg of L^{-1} of extract of *S. acmella* was enough to promote fast (induction time within 3 min) and deep anaesthesia in juvenile tambaqui (46.6 g), which were also evaluated for secondary stress responses post-anaesthesia during 72 hours in recovery. Transient physiological changes occurred mainly between 2 and 24 h post-anaesthesia with *S. acmella* extract and variables returned to normal values after 72 h in recovery, except for the lower concentration of blood Na^+ which did not return to normal levels compared to reference values of non-anaesthetized fish. Tricaine efficiently induced fast and deep anaesthesia at 240 mg L^{-1} and forward and no clear advantages were observed with the use tricaine in transports for up to 10 h at the sedation concentration of 20 mg L^{-1} for the mitigation of oxidative stress. The EO *N. grandiflora* required at least 200 µL L^{-1} to promote deep anaesthesia, however, fast induction was not achieved. The extract of *S. acmella* at 10 mg L^{-1} was sufficient to promote fast and deep anaesthesia in juveniles (3.3 g) subjected to the anaesthetic baths. After transport for 2, 6 and 10 h in the presence or absence of EO of *N. grandiflora* and extract of *S. acmella* (30 µL L^{-1} L and 1 mg L^{-1}, respectively) tissues (muscle, liver, brain and gills) were collected and evaluated for oxidative stress responses throught the indicators: total antioxidant capacity (ACAP), GST activity and thiobarbituric acid reactive substances (TBARS). These concentrations of EO *N. grandiflora* and extract of *S. acmella* were effective in protecting tissues against oxidative damage mainly in muscle and gills of transported fish. Juvenile transported for 2, 6 and 10 h in the presence of *S. acmella* extract (1 mg L^{-1}) and tricaine (20 mg L^{-1}) showed no significant changes in secondary stress responses in blood compared to animals transported without anaesthetics, whereas the EO of *N. grandiflora* at the concentration of 30 mg L^{-1} restrained these responses mainly by decreasing or maintaining normal blood glucose levels and maintaining normal concentrations of Na^+ in blood after transport. All anaesthetics used in this study were effective and safe to promote deep anaesthesia and uneventful recovery in tambaqui. Sedation concentrations of the extracts of *N. grandiflora* and *S. acmella* are therefore recommended for juvenile tambaqui transported in plastic bags for up to 10 h since secondary stress responses were attenuated and lipoperoxidation process in tissues was reduced by the presence thereof.

Key–words: Anaesthesia, oxidative stress, transport, MS-222, *Spilanthes acmella*, *Nectandra grandiflora.*

1. INTRODUÇÃO

1.1. Produção em aquicultura

A produção da aquicultura mundial, incluindo quase 24 milhões de toneladas de plantas aquáticas, alcançou a marca de 90,4 milhões de toneladas em 2012. Desse total, a América Latina produziu cerca de 2,5 milhões de toneladas (FAO, 2014).

O Brasil produziu aproximadamente 2 milhões de toneladas de pescado segundo dados preliminares de 2013 do Ministério da Pesca e Aquicultura (MPA) sendo que 40% desse total foi oriundo de atividades de aquicultura que entre os anos de 2003 e 2009 tiveram um incremento de produção de 35%. O país produziu no ano de 2011 aproximadamente 620 mil toneladas em aquicultura (MPA, 2011). Apesar do incremento significativo na aquicultura nacional e, sem levar em conta a produção de plantas aquáticas e produtos não alimentícios, o Brasil contribuiu com somente 1,1% da produção mundial em aquicultura em 2012 (FAO, 2014). De todo modo, a produção aquícola nacional de origem continental, modalidade que corresponde a aproximadamente 86% da aquicultura total nacional, aumentou de forma significativa de 2010 para 2011, com um incremento de aproximadamente 38% (MPA, 2011).

Na região amazônica existem diversas espécies dulcícolas com potencial para a piscicultura e a atividade encontra-se em estágio de desenvolvimento muito embora já esteja relativamente bem desenvolvida em outras regiões do país (Saint-Paul, 1986; Val & Honczaryk, 1995; Val et al., 2000). O Estado do Pará, por exemplo, apesar do potencial que apresenta para a aquicultura responde por cerca de somente 1,6% da produção anual em aquicultura no país. Segundo dados do Boletim do MPA de 2011 o Pará está à frente somente dos Estados do Acre e Amapá em termos de produção em cativeiro de organismos aquáticos na região amazônica.

O tambaqui (*Colossoma macropomum,* Cuvier 1818) representa aproximadamente 20,5% de toda produção da aquicultura continental nacional e 53% das 206,7 mil toneladas de peixes redondos produzidos em cativeiro no país sendo a principal espécie criada na região norte (MPA, 2011). O *C. macropomum* é o maior Characiformes da Bacia Amazônica e com ampla distribuição na América do Sul (Goulding, 1980). É onívoro na fase juvenil e predominantemente frugívoro em

ambiente natural quando adulto (Freeman, 1995). Pode atingir na natureza até 1 metro de comprimento total e 30 kg de peso. Possui carne bastante apreciada e apresenta-se sobre explorado no ambiente natural, sendo esses os fatores que impulsionaram seu uso na piscicultura (Goulding & Carvalho, 1982; Goulding, 1993; Araújo-Lima & Goulding, 1998).

O tambaqui também é conhecido como cachama, gamitana e black pacu; habita águas ricas em nutrientes, de pH entre 4 e 6 e temperaturas entre 25 a 34 ^{o}C. É uma espécie que consegue sobreviver por longos períodos em águas com níveis baixos de oxigênio (0,5 a 1 mg L^{-1}), em parte, devido a adaptações morfológicas de expansão do lábio inferior, que aumentam a captação de oxigênio da superfície da água quando em condições de hipóxia (Saint-Paul, 1988; Val et al.*,*1998).

As larvas desse peixe se alimentam de zooplâncton e pequenos invertebrados enquanto que os juvenis e adultos podem se alimentar de sementes, frutas, invertebrados e outros animais. Os adultos têm sua primeira maturação entre 3-4 anos de idade e as fêmeas são capazes de produzir em torno de 78 ovos/grama de peso vivo, realizando suas desovas entre outubro e fevereiro (Araújo-Lima & Goulding, 1998; Baldisserotto & Gomes, 2005). A espécie é bem adaptada às condições de cativeiro, aceitando rações artificiais, apresenta alta produtividade, rusticidade, índices desejáveis de crescimento e conversão alimentar (Araújo-Lima & Goulding, 1998; Inoue et al*.,* 2011).

1.2. A síndrome estresse

O estresse está sempre presente na piscicultura como consequência do manejo e pode ser definido como uma condição em que a homeostase é ameaçada ou perturbada em decorrência da ação de estímulos denominados estressores (Wendeelar-Bonga, 1997). Estressores produzem efeitos que ameaçam ou perturbam o equilíbrio homeostático e também provocam um conjunto de respostas comportamentais e fisiológicas como ação compensatória e/ou adaptativa, habilitando o animal para superar as ameaças. Se um animal está submetido a estresse intenso e constante, a resposta fisiológica pode perder seu valor adaptativo e tornar-se disfuncional, acarretando danos permanentes à sua saúde e bem-estar (Carmichael, 1984). O aspecto central da adaptação ao estresse é a realocação de energia para longe de atividades de

alta demanda energética, como crescimento e reprodução, em direção às atividades que promovam restauração da homeostase, tais como respiração, locomoção, balanço hidromineral e reparação de tecidos. Tal dinâmica pode ser refletida negativamente na capacidade de desempenho do peixe a curto ou longo prazos no caso de exposição a situações de estresse agudo ou crônico, respectivamente (Schreck, 1981; Schreck, 1990; Kebus et al., 1992; Pankhursk & Kraak, 1997; Mommsen et al., 1999).

A resposta ao estresse em peixes é um evento integrado, entretanto é comum fazer-se didaticamente a distinção entre resposta primária, secundária e terciária (Pickering, 1981; Wedemeyer et al., 1990; Pickering & Pottinger, 1995; Barton, 2002). A resposta primária compreende a ativação dos centros cerebrais, resultando em massiva liberação de catecolaminas (adrenalina e noradrenalina) e corticosteróides (cortisol), enquanto que a resposta secundária é usualmente definida como a canalização das ações e dos efeitos imediatos desses hormônios em nível sangüíneo e de tecidos, incluindo o aumento dos batimentos cardíacos e da absorção de oxigênio, e a mobilização de substratos de energia e, ainda, a perturbação do balanço hidromineral. A resposta terciária manifesta-se em nível de população traduzindo-se em inibição da reprodução (Small, 2004), do crescimento e da resposta imune (Weendelar-Bonga, 1997).

Práticas comuns no manejo de peixes podem desencadear estresse e como resposta os animais apresentam alterações bioquímicas e fisiológicas que podem aparecer em segundos e persistir por horas ou dias. Eventuais desequilíbrios nos parâmetros de qualidade da água (concentração de oxigênio dissolvido, pH, temperatura, salinidade, elevadas concentrações de amônia e de CO_2, bem como a presença de poluentes) também são causa de estresse (Smart, 1981; Yadav & Akela, 1993; Alkahem, 1994). Operações de rotina muitas vezes induzem a uma resposta de estresse fisiológico que pode ocasionar resultados indesejáveis, tais como a imunossupressão e retardo do crescimento (Roubach et al., 2005; Heo & Shin, 2010).

Diversos indicadores são utilizados para avaliar a intensidade do estresse fisiológico em peixes, tais como a concentração plasmática de cortisol, catecolaminas, glicose, lactato, lipídio, eletrólitos, proteínas, hemoglobina, além do hematócrito e do leucograma (Pickford et al., 1971abc; Mazeaud et al., 1977; Tomasso et al., 1980; Davis & Parker, 1986; Robertson et al., 1987).

1.3. Estresse oxidativo

A perturbação do equilíbrio entre agentes pró-oxidantes e antioxidantes nos tecidos pode ocasionar elevação de danos em proteínas celulares, lipídios, ácidos nucléicos, entre outras estruturas moleculares e, quando esse desequilíbrio está a favor de uma condição pró-oxidante estabelece-se uma situação de estresse oxidativo (Halliwel & Gutteridge, 1999; Bisbal et al., 2010).

As espécies reativas de oxigênio (ERO), como o ânion superóxido (O_2^-), radical hidroxila (HO^-) e o peróxido de hidrogênio (H_2O_2) são produzidas no organismo em condições fisiológicas normais ou patológicas (Wajner et al., 2004). A necessidade de prevenir e/ou interceptar ERO, no decorrer da evolução, proporcionou o desenvolvimento de defesas antioxidantes, comumente divididas em enzimáticas e não-enzimáticas. Dentre as defesas antioxidantes não-enzimáticas, o tripeptídeo glutationa (GSH) é considerado a primeira ação de defesa contra as ERO (Anderson, 1998; Dickinson & Forman, 2002). Outros antioxidantes não enzimáticos como o α-tocoferol, carotenóides e flavonóides interferem na ação das ERO ou regeneram danos causados a sistemas biológicos essenciais (Barreiros et al., 2006).

A enzima glutationa-S-transferase (GST) catalisa reações de conjugação de grande variedade de xenobióticos com o tripeptídeo glutationa (GSH) em um processo que ajuda na detoxificação celular (Habig et al., 1974). A GST pertence a um grupo de enzimas multifuncionais envolvidas na biotransformação e detoxificação de xenobióticos, sua grande característica é proteger as células auxiliando na detoxificação contra danos oxidativos e produtos derivados de processos peroxidativos (Van der Oost et al., 2003). Os antioxidantes enzimáticos protegem os organismos quando proporcionam a decomposição de radical ânion superóxido (O_2^-) ou ainda a degradação de agentes pró-oxidantes menos reativos como o peróxido de hidrogênio (H_2O_2). Desta forma, a análise da atividade enzimática é indicada para aferição da competência antioxidante em animais (Huber et al., 2008).

Em razão da intensificação da piscicultura, procedimentos ou insumos que reduzam os índices de mortalidade, elevem as taxas de crescimento, aumentem a resistência dos animais ao estresse do manejo e atenuem o estresse oxidativo têm sido

alvos de pesquisa (Pickering, 1992; Wendelaar Bonga, 1997; Barnett & Pankhurst, 1998; Azambuja et al., 2011).

1.4. Utilização de anestésicos na aquicultura

No intuito de refrear os efeitos deletérios ocasionados pelo estresse de manejo, a utilização de produtos anestésicos vem se intensificando na aquicultura moderna e tem relevância prática, facilitando o manuseio em diversas situações onde houver necessidade de manipulação dos animais, como ocorre nas amostragens periódicas do plantel, na marcação dos animais, no transporte, na reprodução artificial e ainda nos procedimentos cirúrgicos (Coyle et al., 2004; Roubach et al., 2005; Weber et al., 2009).

A escolha de um anestésico apropriado depende principalmente da sua eficácia de indução e imobilização com rápida recuperação (Gilderhus & Marking, 1987; Burka et al., 1997; Ross & Ross, 2008). Um anestésico ideal deve possuir vários atributos, tais como: ser atóxico, de baixo custo, de fácil administração e resultar em rápida indução e recuperação calma (Treves-Brown, 2000). A atividade de natação, o equilíbrio, o comportamento, a taxa de ventilação, o movimento dos olhos, as respostas de reflexo e da frequência cardíaca são parâmetros monitoráveis em peixes. O grau de atividade desejado será dependente do aprofundamento do nível de anestesia para um procedimento particular (Sneddon, 2012).

A eficácia anestésica também é condicionada pelo ambiente (temperatura, pH e salinidade) e fatores biológicos (tamanho, peso, teor de lípidos e espécies de peixes) (Burka et al., 1997; Ross & Ross, 2008). É bastante aconselhável identificar as mais baixas concentrações eficazes em proporcionar o plano anestésico almejado, sendo que as respostas a um mesmo anestésico podem variar consideravelmente entre as diferentes espécies (King et al., 2005).

O uso de anestésicos pode reduzir os danos ocasionados pelo estresse atenuando a resposta fisiológica ao mesmo (Weber et al., 2009). Uma série de substâncias químicas revelaram-se eficazes na anestesia de peixes, cada uma com seus próprios méritos e deméritos (Velíšek et al., 2006). A anestesia pode ser induzida através de banho, por via oral, ou através de injeção com uma variedade de substâncias. Os anestésicos mais utilizados na aquicultura são a tricaina metanossulfonato (MS-222) e o

2 - fenoxietanol (PE) (Svoboda & Kolarova, 1999), além do etanol, éter dietílico, benzodiazepinas, halotano, lidocaína, cetamina, medetomidina, propofol, dióxido de carbono e oxigênio (Neiffer & Stamper, 2009; Weber III, 2011). Outros estudos têm avaliado a eficácia anestésica da benzocaína (etil aminobenzoato) que também tem sido utilizada com frequência em diferentes espécies de teleósteos (Heo & Shin, 2010; Pramod et al., 2010).

Anestésicos provenientes de fontes naturais como óleos essenciais de plantas podem ser uma importante área de pesquisa em razão da grande diversidade de compostos presentes nesses produtos (Keene et al*.,* 1998; Gonçalves et al*.,* 2008; Cunha et al*.,* 2010; 2011). O óleo de cravo, que tem por principal componente ativo (70% a 90%) o eugenol [2-metoxi-4-(2-propenil) fenol] tem sido investigado em diversos estudos e qualifica-se como um produto natural eficiente para indução anestésica a um bom custo-benefício (Walsh & Pease, 2002; Iversen et al., 2003; King et al., 2005; Mylonas et al., 2005; Roubach et al., 2005; Cunha & Rosa, 2006; Hajek et al., 2006; Barbosa et al., 2007).

Outros produtos naturais apresentam potencial para utilização como anestésicos na aquicultura. Gonçalves et al. (2008) avaliaram o mentol para anestesiar juvenis de pacu, *Piaractus mesopotamicus* e ele se mostrou eficiente para promover a indução anestésica. Extratos de óleos essencias das plantas erva cidreira *Lippia alba* e a alfavaca ou manjericão *Ocimum gratissimum* também foram testados em jundiá, *Rhamdia quelen,* mostrando-se efetivos e seguros para essa espécie. O óleo essencial de *L. alba* amenizou inclusive o estresse oxidativo em experimentos de transporte simulado (Cunha et al., 2010; Azambuja et al., 2011; Silva et al., 2012).

Além da *L. alba*, outro óleo essencial, o da planta *Aloysia triphylla,* foi testado no crustáceo *Litopenaeus vannamei,* sendo que ambos foram eficazes na indução anestésica em plano mais profundo e em concentrações mais baixas para obtenção de planos mais superficiais de anestesia nos experimentos de transporte simulado (Parodi et al., 2012).

O jambu (*Spilanthes acmella* var *oleracea L.*), também conhecido como agrião-do-Pará, cresson du Pará, dentre outros, é uma hortaliça de clima tropical, nativa do Brasil, cultivada ao longo do ano como planta ornamental ou medicinal (Prachayasittikul et al., 2013). Extrato etanólico das folhas de *S. acmella* foram

investigadas e ficou demonstrada atividade anti-inflamatória significativa em processos inflamatórios agudos, subagudos e crônicos, bem como atividade analgésica central e periférica em modelos experimentais animais (Barman et al., 2009). Outro trabalho demonstrou que o extrato etanólico de *S. acmella* produz efeitos antinociceptivos prevalentes e não causa efeitos adversos. A presença de N-alquilamidas, incluindo o espilantol, sugere que o efeito terapêutico está relacionado com a sua maior atividade anestésica (Nomura et al., 2013).

O gênero *Nectandra* (Lauraceae) está distribuído por todo o Brasil até o Uruguai (Alves & Sartori, 2009). É um grupo de plantas que tem sido usado em medicina popular como produtos analgésicos (Santos Filho & Gilbert, 1975), sedativos (Alves et al., 2008) e anti-inflamatórios (da Silva et al., 2004; Garcez et al., 2009). Os extratos de canela-amarela *Nectandra grandiflora* Nees apresentam alcalóides com propriedades anti-tumorais (Moreno et al., 1993) e propriedades antioxidantes já foram descritas nos extratos obtidos das folhas (Ribeiro et al., 2005).

Alguns agentes anestésicos nem sempre são eficazes para peixes, sendo possível, paradoxalmente, que aumentem os níveis de estresse, potencializando a liberação de catecolaminas, ou que não tenham efeito nenhum efeito mitigador sobre a resposta de estresse fisiológico ocasionado pelos diversos estressores do manejo rotineiro (Bressler & Ron, 2004; Zahl et al., 2010; Weber III, 2011).

Não obstante esses efeitos negativos, via de regra, a anestesia produz uma resposta de menor estresse em relação ao manuseio e transporte livre de fármacos, com base na comparação dos níveis de cortisol em circulação, bem como indicadores secundários, tais como glicose no sangue, hematócrito, hemoglobina, lactato e osmolalidade (Bressler & Ron 2004; Small, 2005; Crosby, et al., 2006).

A anestesia superficial, também denominada de sedação, pode ser suficiente para diminuir o estresse no transporte de peixes, visto que este não é um procedimento invasivo (Sneddon, 2012). Com a utilização de anestésicos em concentrações mais baixas ocorre perda parcial de equilíbrio e supressão da reação a estímulos externos. É considerada uma condição ideal para o transporte de peixes que apresentam, nessas condições, atividade reduzida, mas são capazes de manter o equilíbrio parcial, a capacidade de nadar e evitar danos físicos resultantes da colisão com sacos de plástico (Cooke et al., 2004).

Nos estudos de Inoue et al. (2005), matrinxãs *Brycon cephalus* foram expostas ao eugenol na concentração de 5 mg L^{-1} em sacos de polietileno em experimento de transporte e como resultado houve sedação branda e mitigação dos efeitos do estresse fisiológico.

A forma mais usual de transporte de alevinos no Brasil se dá por sistemas fechados em sacos de polietileno, que são inflados com oxigênio puro, havendo por consequência bruscos aumentos nos níveis de oxigênio dissolvido (Gomes et al., 1999; Golombieski et al., 2003). Sendo o transporte um ponto crítico da cadeia produtiva da piscicultura e também um agente estressor, a exposição de peixes inicialmente à hiperóxia e posteriormente à hipóxia/anóxia no decorrer do transporte, pode resultar em alterações oxidativas, pois o consumo de oxigênio determina os níveis de ERO gerados e também o status antioxidante (Wilhelm Filho et al., 2001; 2002). Alguns indicativos podem ser dados pelo aumento das atividades de enzimas antioxidantes que sofrem um incremento de atividade com a elevação nos níveis intracelulares de ERO (Lushchak et al., 2001).

Peixes que são submetidos ao transporte e que sejam mais adaptados às variações nos níveis de oxigênio dissolvido à hipóxia ou anóxia, sobrevivem com mais frequência às condições de baixo oxigênio dissolvido, entretanto, sofrem um novo perigo após a retomada do oxigênio. A cadeia transportadora de elétrons ao ser reduzida sob condições de hipóxia pode produzir níveis elevados de ERO durante a reoxigenação o que pode causar estresse oxidativo. A hiperóxia por si, normalmente estabelecida no início do transporte de peixes em sistemas fechados pela introdução de oxigênio puro, pode gerar níveis elevados de ERO e esses animais precisam desenvolver sistemas antioxidantes eficazes. Uma forma de aliviar o estresse oxidativo ocasionado durante o transporte de jundiá, *Rhamdia quelen* foi utilizando o óleo essencial de *L. alba* na concentração de 10 μL L^{-1}, o qual demonstrou além de atividade anestésica, propriedade antioxidante (Azambuja et al., 2011).

Ademais, o uso de produtos naturais é potencialmente menos oneroso e menos problemático quanto a questão residual que comprometa a qualidade da água ou da carne. A utilização de anestésicos vem se tornando sistemática à medida que a piscicultura se intensifica no Brasil. É importante que seja investigada a eficácia dos diversos agentes anestésicos para as diferentes espécies, mesmo os de eficácia

inquestionavelmente estabelecida como no caso da tricaína (MS-222), uma vez que as respostas à indução anestésica pelo mesmo produto são bastante variáveis entre espécies.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a eficácia de extratos vegetais e da tricaína (MS-222) para anestesia, sedação e transporte de juvenis de tambaqui *Colossoma macropomum* e suas implicações sobre as respostas de estresse.

2.2. Objetivos específicos

- Verificar a eficácia anestésica de extrado de jambu *Spilanthes acmella* e as respostas secundárias de estresse pós-anestesia no sangue de juvenis de tambaqui *C. macropomum* em diferentes tempos na recuperação;
- Determinar a concentração ideal de tricaína (MS-222) para anestesia e as respostas de estresse oxidativo através da verificação da capacidade antioxidante total (ACAP), da peroxidação lipídica (POL) e da atividade da glutationa-S-transferase (GST) em amostras de brânquias, fígado e cérebro de juvenis de *C. macropomum*;
- Avaliar a atividade anestésica do óleo essencial de canela-amarela *Nectandra grandiflora* e do extrato de jambu *S. acmella*, bem como o estresse oxidativo, através da verificação da capacidade antioxidante total (ACAP), da peroxidação lipídica (POL) e da atividade da glutationa-S-transferase (GST) em amostras de músculo, brânquias, fígado e cérebro de juvenis de *C. macropomum* submetidos à sedação e diferentes tempos de transporte;
- Estudar o efeito da presença de anestésicos (extrato de jambu, MS-222 e óleo essencial de canela-amarela) sobre as respostas secundárias de estresse no pós transporte de juvenis de tambaqui *C. macropomum*.

3. HIPÓTESES

- O extrato de jambu *S. acmella*, o óleo essencial de canela-amarela *N. grandiflora* e a tricaína (MS-222) são produtos anestésicos eficazes para juvenis de tambaqui.

- Concentrações de sedação de extrato de jambu *S. acmella*, de óleo essencial de canela-amarela *N. grandiflora* e de tricaína (MS-222) atenuam respostas secundárias de estresse, inclusive estresse oxidativo em juvenis de tambaqui submetidos à transporte em condições de hiperóxia.

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CAPÍTULO 1

Jambu, *Spilanthes acmella* as a novel anaesthetic for juvenile tambaqui, *Colossoma macropomum*: secondary stress responses during recovery

Running title: Extract of jambu as anaesthetic for fish

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Abstract

The aim of this study was to evaluate the efficacy of the extract of jambu flowers, *Spilanthes acmella* as a new anaesthetic for fish, using juvenile tambaqui *Colossoma macropomum* (Cuvier, 1818) as a model. It was evaluated the time to induction and recovery by dose-response trials, and secondary stress responses after short-term anaesthesia. Juveniles were placed in aquaria containing five different concentrations of jambu extract (5, 10, 15, 20, 25 mg L^{-1}) and the times of anaesthetic induction and recovery were determined. Sham control fish and animals exposed to ethanol-added water were used as controls. The secondary stress responses of fish following anaesthesia with jambu extract (20 mg L^{-1}) were investigated through assessment of whole blood variables: glucose, ions (Na^+ K^+, and Ca^{++}), osmolality, Htc, Hb, partial pressures of CO_2 (pCO_2) and O_2 (pO_2), bicarbonate concentration (HCO_3^-), and pH). Deep anaesthesia was observed at all concentrations tested in this study. The use of 20 mg L^{-1} of this compound is recommended for rapid induction (<3 min) and uneventful recovery (<5 min) from deep anaesthesia; while the concentration of 2 mg L^{-1} is sufficient to promote slight sedation. Only transient changes in secondary stress responses were observed in tambaqui during recovery, with most of the parameters returning to initial values within 48h post-anaesthesia. Therefore, the extract of jambu flowers can be considered an efficient anaesthetic for tambaqui, and its use for other fish species should also be considered.

Keywords: Anaesthesia, sedation, fish, aversion, blood parameters, Brazil cress

1. Introduction

Anaesthetics are important in aquaculture to reduce handling stress and mortality and have also been used for research and veterinary medicine purposes (Sneddon, 2012). In addition to the advantages of using anaesthetics to mitigate stress and decrease mortality rates, recent research studies have raised awareness about the importance of using anaesthetics for aquatic organisms also from an ethical perspective. Since there is reasonable evidence that fish are capable of nociception or pain perception (Ashley et al., 2007; Roques et al., 2010), welfare and pain are important aspects to be addressed.

The osmoregulatory and electrolytic alterations caused by stress in aquatic animals are typical changes of fish under adverse conditions. Stressful environmental conditions cause passive ion loss and water influx in fish maintained in hypo-osmotic environments, thus affecting negatively the function of active exchange mechanisms in teleosts (Wendelaar Bonga, 1997).

A number of chemicals are used as fish anaesthetics, but most of the commonly used anaesthetics, *e.g.* tricaine methane-sulphonate (MS-222), quinaldine and 2-phenoxy-ethanol have been regarded as aversive, toxic and expensive (Mgbenka & Ejiofor 1998; Roubach et al., 2001; Readman et al., 2013).

For many countries, the use of fish anaesthetics is a matter of concern, since there are no specific laws regulating the use of such drugs. The recommendations of the US Food and Drug Administration (FDA) are usually followed. Anaesthetic acquisitions can be also difficult, *e.g.* MS-222 is the only FDA-approved anaesthetic for food fish in the USA and is not produced or accessible in many countries (Gomes et al., 2001). Benzocaine is one of the most used anaesthetics in Latin America and results obtained by Gomes et al. (2001) with tambaqui *Colossoma macropomum* (Cuvier) juveniles suggest that it meets most of the criteria established for an ideal fish anaesthetic. Yet, no legislation exists as to the use of benzocaine in this region.

Compounds from natural sources such as extracts of plants are promising alternatives to traditional synthetic drugs, since plants may be rich in chemicals with biological implications, for instance, anaesthetic/analgesic activities. Moreover, natural compounds may be as effective, safe or even cheaper when compared to current traditional chemicals used for fish anaesthesia (Keene et al., 1998; Gonçalves et al., 2008; Cunha et al., 2010).

Alternative anaesthetics such as menthol and eugenol have been previously tested on juvenile tambaqui (Façanha & Gomes, 2005; Roubach et al., 2005), being the latter largely used for other tropical teleosts species (Vidal et al., 2007; Vidal et al., 2008; Inoue et al., 2011). Essential oils of *Lippia alba*, *Ocimum gratissimum* and *Aloysia triphylla* have been recently investigated for their anaesthetic properties and were presented as new natural compounds for anaesthesia of aquatic organisms (Cunha et al., 2010; Azambuja et al., 2011; Benovit et al., 2012; Parodi et al., 2012; Silva et al., 2012).

The Genus *Spilanthes* is comprised of approximately 60 species widely distributed over tropical and subtropical regions of the world, namely in Africa, America and Asia (Chung et al., 2008; Tiwari et al., 2011). *Spilanthes acmella* var *oleracea* is commonly known as jambú, toothache plant, Brazil cress and Para-cress. This is a plant original from the tropics of Asia and South America (Chung et al., 2008; Prachayasittikul et al., 2013). This species is for long used in traditional cuisines and medicines of different civilizations (Hind & Biggs, 2003).

Previous studies have assessed *S. acmella* extract bioactivity as analgesic and local anaesthetic in rats and mice (Ansari et al., 1988; Chakraborty et al., 2002; Nomura et al., 2013). Leaves of the plant were investigated and significant anti-inflamatory activity was observed in acute and chronic inflammatory processes, as well as central and peripheral analgesic activities in experimental animal models (Barman et al., 2009). Other studies report the plant extracts also proved to possess anti-nociceptive activity against continuous inflammatory pain and anti-hyperalgesic activity, possibly by inhibiting prostaglandin synthesis (Ratnasooriya & Pieris 2005) showing no side effects. The presence of N-alkylamides in jambu, namely "spilanthol" (N-isobutyl-2E,6Z,8E-decatrienamide) suggests that the therapeutic effect observed is a result of its marked anaesthetic activity (Nomura et al., 2013). Spilanthol can be concentrated through conventional extraction solvent methods (ethanol), which has once been found to contain 9.04% of total N-alkylamides yet 88.84% spilanthol (Boonen et al., 2010). Furthermore, highly efficient extraction methods have been described such as the supercritical fluid extraction [SFE(CO_2)], yielding 65.4% and 47.3% of spilanthol from jambu flowers and leaves respectively (Dias et al., 2012).

Frequently, the use of anaesthetics to reduce handling stress in fish is advantageous based on comparisons of plasma cortisol levels, glycemia, plasma lactate concentration, haematology, and osmolality between anaesthetized and non-anaesthetized animals (Bressler & Ron 2004; Small, 2005; Crosby, et al*.,* 2006). Contradictorily, anaesthetics themselves can also be aversive and induce stress responses by potencializing catecholamine release into the blood stream (Bressler & Ron 2004; Zahl et al., 2010; Weber III, 2011). It has been reported that MS-222 caused a ‘detrimental physiological impact’ in silver catfish, *Rhamdia quelen* (Gressler et al., 2014).

Tambaqui *C. macropomum* is the most widely farmed native fish species in Brazil and in several other countries in South and Central America (FAO, 2014). Therefore, this species has the potential to be selected as a model to establish studies with anaesthetics for tropical species. To date, there are no reports about sedative and/or anaesthetic efficacy of jambu, *S. acmella* on fish. The aim of this study was to evaluate the anaesthetic activity of jambu on juvenile tambaqui, through determination of time to induction and recovery by dose-response trials, and secondary stress responses after short-term anaesthesia.

2. Materials and Methods

2.1. Animals

Tambaqui juveniles were purchased from a commercial fish farm in Brazilian Amazon and transported to the laboratory where they were maintained in continuously aerated 250 L tanks, with controlled water parameters. Fish were acclimated for 15 days in two recirculation systems prior to the beginning of the experiments. Photoperiod was fixed at 12 L/12 D. Fish were fed twice a day at 2% of biomass with commercial feed (28% crude protein). Juveniles were fasted for a period of 24 h prior to the experiments. The experiments were approved by the Ethical and Animal Welfare Committee of the of the Universidade Federal do Rio Grande - FURG.

2.2. Water Quality

The parameters (mean ± S.D) were maintained as follows: Dissolved Oxygen (DO) (experiment 1: 6.45 ± 0.39 mg L^{-1}; experiment 2: 6.82 ± 0.09 mg L^{-1}) and temperature (experiment 1: 25.6 ± 0.7 °C; experiment 2: 25.7 ± 0.1 °C) were measured using an oxygen meter (Yellow Springs Instruments, Yellow Springs, OH, USA); pH (experiment 1: 7.1 ± 0.2; experiment 2: 7.05 ± 0.1) was determined with a Five Easy FE20, Switzerland. Total ammonia nitrogen (TAN) (experiment 1: 0.80 ± 0.08 mg L^{-1} $NH_4^+ + NH_3^-$ - N; experiment 2: 0.90 ± 0.05 mg L^{-1} $NH_4^+ + NH_3^-$ - N) was quantified according to Unesco (1983), and nitrite was determined according to Bendschneider &

Robinson (1952) (experiment 1: 0.03 ± 0.01 mg L^{-1}; experiment 2: 0.02 ± 0.01 mg L^{-1}). Total alkalinity was evaluated by titration in accordance with Eaton et al. (2005) guidelines (experiment 1: 41.7 ± 0.09; experiment 2: 52.6 ± 0.05 mg $CaCO_3$ L^{-1}, respectively).

2.3. Plant Materials

Samples of the extract of the flowers of *Spilanthes acmella* var *oleracea* (L.) were obtained by means of fractionated supercritical fluid extraction methodology with the use of CO_2 [SFE(CO_2)] to remove less polar compounds from the flowers of the plant. For details on the methodology of extraction and chemical composition of the extract see Dias et al. (2012).

Vegetal oil extracts, in the form of a wax, such as this obtained from jambu flowers through [SFE(CO_2)] extraction methodology are poorly diluted in water and therefore it is necessary a pre-dilution in ethanolic vehicle before using in anaesthetic baths for fish. The stock solution of *S. acmella* extract was prepared by weighing and diluting the jambu flowers extract in comercial alcohol (96%) yielding a 2.24g L^{-1} solution which was stored in an amber glass bottle at 4°C until its use.

2.4. Biological activity

2.4.1. Experiment 1: Anaesthetic efficacy of extract of jambu, *S. acmella*.

Juvenile fish (46.6 ± 6.2 g; 14.6 ± 0.8 cm, total length) were transferred to aquaria containing 30 L of continuously aerated water. Concentrations of the extract at 5, 10, 15, 20 and 25 mg L^{-1} were used in this experiment. A sham control group was used and animals (n = 5) were transferred to aquaria with anaesthetic-free water and observed for 30 min. A vehicle control added with the same volume of ethanol to reach the concentration of 25 mg L^{-1} of jambu extract in the water was also evaluated. In order to evaluate the cumulative time required to reach the different stages of induction to and recovery from anaesthesia a digital stopwatch was used. Groups of 10 juveniles were used for each concentration tested and each juvenile was observed individually and

considered a replicate, they were used only once. All animals were starved for 24h prior to the tests. The characterization of the induction and recovery stages was considered as described by Park et al. (2008) with modifications and is shown in Table 1.

The maximum observation time was 30 min. After induction, juveniles were transferred to tanks with anaesthetic-free water, and the time elapsed for recovery was registered. Animals were considered to have recovered when they showed normal swimming behaviour. After recovery, fish were grouped according to the anaesthetic concentration and transferred to continuously aerated 250 L tanks, where they were observed for 48 hours to check for mortalities.

In order to investigate the effectiveness of a sedation state (slight anaesthesia), a group of fish (n = 10) was exposed for 10 min to 2 mg L^{-1} of jambu extract which corresponded to 10% of the anaesthetic concentration (20 mg L^{-1}) considered adequate to induce deep anaesthesia of juvenile tambaqui.

2.4.2. Experiment 2: Evaluation of secondary stress responses.

This experiment was conducted to verify stress response of fish in recovery after short-term exposure (3 min) to *S. acmella* extract at the concentration of 20 mg L^{-1}. Tambaqui juveniles (50.5 ± 3.9 g; 15.0 ± 0.4 cm, total length) were assayed in five groups (n = 10 per sampling time post-anaesthesia). A sham control group (CT) and an ethanol control (EC) were used (n = 10 in each control group). In the the EC fish were exposed to water added with ethanol at the same volume used to provide the concentration of 20 mg L^{-1} of jambu extract.

Fish were captured with a dip net and individually transferred to continuously aerated 30 L aquaria previously added with jambu extract at 20 mg L^{-1} where they were exposed to short-term anaesthetic baths of 3 min. Immediately following anaesthesia, all fish were handled for biometric measurements and transferred to their respective anaesthetic-free 100 L recovery tanks. Similarly, CT and EC groups were transferred to identical aquaria and were also maintained for 3 min in their respective anaesthetic-free water tanks, simulating the same handling procedures of anaesthetized fish.

Secondary stress responses were followed for 72 h after fish were anaesthetized. Blood samples (300-500 μL) were taken from the caudal vasculature with 1 mL syringes coated with heparin at 0 (immediately after recovery), 2, 24, 48, and 72 h.

Whole blood was used to determine glucose, ionic concentration (Na^+, K^+, and Ca^{++}), hematocrit (Htc), hemoglobin (Hb), partial pressure of CO_2 (pCO_2), partial pressure of O_2 (pO_2), pH, and bicarbonate concentration (HCO_3^-) using a portable i-STAT® clinical analyzer with CG8+ cartridges (Abbott laboratories, Chicago, IL, USA). The values obtained for pCO_2, pO_2, pH and HCO_3^- were corrected for the water temperature (25°C) according to the manufacturer's instructions and also as described by Hanley et al. (2010). The use of i-STAT® and calculations for blood gases have been described for several fish species (Jacobs et al., 1993; Pidetcha et al., 2000; Harrenstien et al., 2005; Kristensen et al., 2010). Blood osmolality was determined using a vapor-pressure osmometer (Vapro 5520; Wescor, Inc.; Logan, Utah, USA).

2.5. Statistical analysis

All data are presented as mean ± SD. To verify the homogeneity of variances and normality, data were submitted to Levene and Kolmogorov-Smirnov tests, respectively. Statistical differences between controls (CT and EC) were tested by Student's t-test ($p<0.05$). The results obtained for stages A1, R2 (experiment 1), pO_2 and Na^+ (experiment 2) were Ln transformed previously to statistical analysis. One-way ANOVA and Tukey test were used for data of anaesthesia induction and recovery, and also for osmolality. One-way ANOVA and Dunnet post-hoc test were used for the other whole blood variables. Trends of the anaesthesia and recovery stages were fitted using non-linear response models with measured times to reach stages being the response variables and concentrations of extract of *S. acmella* the predictor variable. Pearson correlation was performed among blood variables and the minimum significance level was set at $p<0.05$ in all cases.

3. Results

3.1. Short-term anaesthesia induction and recovery

Times of induction and recovery from anaesthesia are show in Table 2. Tambaqui showed a shorter time to reach the stage of agitation (A1) at the concentration of 10 mg L^{-1} and above, compared to the lower concentration used. The agitation behaviour is immediately followed by stage A2 hereby characterized by loss of equilibrium and erratic swimming. The same pattern was noticed for stage A2 and the higher the concentration the faster the behavioural characteristics of this stage were observed, being the time at the concentration of 15 mg L^{-1} or above, significantly shorter to show loss of equilibrium compared to 5 or 10 mg L^{-1}. All the tested concentrations were effective in inducing anaesthesia (stage A3) with loss of reaction to tail pinch stimulus and irregular or minimum opercular beating. Fish exposed to the lowest concentration (5 mg L^{-1}) reached anaesthesia in 7.6 min (459 s) and the subsequent increasing concentrations tested reduced the induction time, the fastest being 2.4 min (144 s) at 25 mg L^{-1}. Based on visual assessment, fish were acknowledged fully recovered after exposure to all concentrations tested.

The overall trends observed as a result of the relation between the time required for anaesthesia or recovery stages over the concentrations tested can be predicted by equations presented in Table 2. Clear decreases ($r^2 = 0.99$, $p<0.05$) in the induction time stages (A1, A2 and A3) occurred with increasing concentrations of jambu extract whereas an increase ($r^2 = 0.74$, $p<0.05$) in the recovery time was observed with increasing concentrations of the extract and were best predicted by power regression. On the other hand, no relation ($p>0.05$) between increasing concentrations of anaesthetic and time to show behaviour associated with stage R1 was present.

Light sedation or superficial anaesthesia was observed when fish were exposed for 10 min to 2 mg L^{-1} of jambu extract.

No mortalities were observed until two weeks after the experiments. Water in sham control group and water comprised solely of ethanol addition at the same volume of the highest concentration of jambu extract used (25 mg L^{-1}) did not produce any anaesthetic effect on tambaqui after 30 min observation.

3.2. Secondary stress responses

No differences were observed between controls (CT and EC) for the secondary stress response in this study ($p>0.05$). There was an increase in blood glucose in juvenile tambaqui following anaesthesia baths with jambu extract at 0 h (74.1 ± 10.47 mg dL^{-1}) and 2 h (73.2 ± 8.05 mg dL^{-1}) and glycemia returned to CT levels (63.3 ± 8.3 mg dL^{-1}) at 72 h after a slight oscillation (Fig. 1).

Blood K^+ and Ca^{++} concentration decreased (2.89 ± 0.44 and 1.20 ± 0.20 mmol L^{-1} respectively) after 2 h in recovery and returned to CT values (3.96 ± 0.39 and 1.47 ± 0.20 mmol L^{-1} respectively) at 24h (Fig. 2A and B). Blood Na^+ concentration (145.3 ± 1.1 mmol L^{-1}) decreased after 2 h not returning to CT levels (150.4 ± 1.3 mmol L^{-1}) after 72 h post-anaesthesia (Fig. 2C). Osmolality was unchanged ($p>0.05$) throughout the recovery period when compared to CT group (294 ± 5.3 mOsm Kg^{-1}) (Fig. 3).

Haematological variables Hct and Hb concentrations showed a consistent biphasic decrease between 2 h (19.0 ± 2.72 % and 6.32 ± 0.91 g dL^{-1} respectively) and 48h (19.0 ± 3.48 % and 6.42 ± 1.18 g dL^{-1} respectively), with both returning to CT values (23.0 ± 2.78 % and 7.80 ± 1.01 g dL^{-1} respectively) at 72 h (Fig. 4A and B).

Partial pressure of oxygen (pO_2) measured in CT (6.99 ± 2.34 mg L^{-1}) did not show significant changes throughout recovery and pCO_2 was decreased between 2-24h (5.78 ± 0.73 and 7.23 ± 0.64 mg L^{-1} respectively) compared to CT (8.76 ± 0.95 mg L^{-1}). However, returned to CT levels at 48 h (Fig. 5A). A slight negative correlation between pO_2 and pCO_2 was observed ($r = -0.36$, $p = 0.003$, Fig. 5A).

A transient decrease was observed in HCO_3^- concentration (4.71 ± 0.6 mmol L^{-1}) in 0h when compared to control (5.65 ± 0.33 mmol L^{-1}) (Fig. 5B). Blood pH was increased ($p<0.05$) after 2h (7.59 ± 0.07) in recovery when compared to CT (7.42 ± 0.05) (Fig. 5C) and was negatively correlated to pCO_2 ($r = -0.84$, $p<0.001$, Fig. 6).

4. Discussion

Anaesthesia induction is often accompanied by hyperactivity, usually a response to the slightly irritant properties of the drug. In general, induction should be rapid and without marked hyperactivity, although there is usually some. The animal will exhibit a

succession of the signs notably ataxia and loss of the righting reflex, eventually passing into surgical anaesthesia with no reaction to any stimuli (Ross & Ross, 2008).

Studies focusing on fish welfare and pain assessment have drawn much attention recently and legislation in Europe as well as researchers have put great emphasis on improving fish welfare (Animals Scientific Procedure Act, 1986; Ashley et al., 2007; EU Directive, 2010; Weber III, 2011; Readman et al., 2013). MS-222 is one of the most commonly used anaesthetic agents in fish research studies and is also used in food fish production (Sneddon, 2012). Concerns, however, have been raised that MS-222 may cause an aversive reaction and irritation of tissues in fish following exposure to the drug (Lewbart, 1998; Williams et al., 2009; Reed & Jennings, 2011); much incidental evidence exists as to the adverse reaction seen in fish during initial stages of anaesthesia (APC, 2009). Wong et al. (2014) exposed zebrafish *Danio rerio* to MS-222 and clove oil and less pronounced aversion was observed for the latter.

The stage of agitation was observed for all concentrations of jambu. Rapid flaring of the opercula, "coughing", surfacing and frantic swimming were present. However, time to the onset of agitation behaviour was concentration dependent and 4-fold faster at the highest concentration (25 mg L^{-1}) compared to the lowest concentration used. The agitation observed suggests transitory distress and aversion to some extent in juvenile tambaqui in this study. Similar behaviour was also observed with tambaqui anaesthetized with benzocaine (Gomes et al., 2001). Although MS-222 is often used as fish anaesthetic, there are no reports on MS-222 anaesthesia in tambaqui.

All five concentrations tested promoted anaesthesia with minimum opercular beating and loss of reaction to tail pinch stimulus (A3) within the stipulated observation time limits. However, only at the concentration of 20 mg L^{-1} of jambu extract, animals reached anaesthesia (A3) in 2.8 min (173 s), therefore complying with the recommended maximum induction time of 3 min (180 s) and recovery within 5 min (300 s) (Bell, 1987; Iwama & Ackerman, 1994; Ross & Ross, 2008).

A variety of anaesthetics have been previously tested for tambaqui with recommended concentrations capable of inducing anaesthesia within 3 min presented in Table 3, and in those cases water quality conditions were quite similar to the conditions verified herein. Nevertheless, great care should be used for comparisons and extrapolation of results to other species. Recent study has demonstrated that although

taxonomic classification is not the main reason for the variations in behaviour response observed during anaesthetic induction and recovery, many other factors are implicated, such as fish size, dissolved oxygen levels and more importantly the concentration used and temperature of the water at the moment of induction (Bowker et al., 2015).

The lowest effective anaesthetic concentration of a compound should always be envisioned for anaesthesia induction. For the correct usage of an anaesthetic, one must know its ideal concentration, since inappropriate concentrations can lead to undesired effects on physiology and also eventually lead to fish mortality. The ideal concentration is also important from an economic perspective, since anaesthetics are expensive and excessive concentrations will increase operating costs (Roubach et al*.,* 2001).

Light sedation was characterized by lethargy and partial loss of equilibrium and reaction to tail pinch stimulus. These conditions are considered appropriate for non-invasive procedures such as biometrics, gill scrapes, tagging, or transport when only sedation is necessary (Sneddon, 2012).

Sladky et al. (2001) verified that fish that had rapid inductions at high concentrations of eugenol were more likely to recover uneventfully. Nevertheless, recovery from anaesthesia can be quite irregular as it was observed in juvenile tambaqui. It has been reported (Ross & Ross, 2008) that lower induction concentrations do not necessarily imply that a faster recovery process from anaesthesia will occur.

Plasma glucose plays an important role in fish metabolism and along with cortisol is one of the most reliable stress responses in fish (Wedemeyer et al., 1990). Rapid increases in plasma glucose are mediated by the release of catecholamines, which increase (presumably in response to the hypoxia caused by cessation of respiration) in the plasma of anaesthetized fish (Gingerich & Drottar, 1989; Iwama et al., 1989).

Our findings are similar to those of Inoue et al. (2011) who observed an increase in plasma glucose in juvenile tambaqui following anaesthesia baths with eugenol at concentrations of 20 and 60 mg L^{-1}. Furthermore, those authors verified that glycemia returned to normal concentrations after 24h when compared to sham control fish, resembling the results of this study despite the slight oscillation observed before returning to CT levels at 72 h. On the other hand, Roubach et al. (2005) reported no differences in glycemia levels of tambaqui over increasing concentrations of eugenol

(35 - 135 mg L^{-1}) or exposure to benzocaine at 100 mg L^{-1}. It is important to notice that those authors did not compare glucose levels with a sham control group.

Benzocaine anaesthesia at 200 mg L^{-1} also increased glycemia levels immediately after recovery in juvenile tambaqui when compared to lower concentrations (50 - 150 mg L^{-1}) (Gomes et al., 2001). Blood glucose concentrations increased in red pacu, *Piaractus brachypomus* exposed to MS-222 or eugenol and increases were also reported even in fish exposed to adequate short-term anaesthetic baths (*i.e.*, < 3 minutes) (Sladky et al., 2001). A variety of anaesthetic drugs tested on several other fish species induced similar patterns with increased glycemia levels following anaesthesia (Ortuno et al., 2002; Deriggi et al., 2006; Barbosa et al., 2007; Park et al., 2008).

No changes were found in the concentration of blood glucose in common carp (*Cyprinus carpio* L.) and European catfish (*Silurus glanis*) following 2-phenoxyethanol (0.3 ml L^{-1}) anaesthesia (Velisek & Svobodova, 2004; Velisek et al., 2007) and conversely, a decreased blood glucose concentration was observed in rainbow trout following MS-222 anaesthesia (Soivio et al., 1977).

Blood glucose concentrations increased rapidly after induction with jambu extract, even after short-term exposure. Despite of this increment in blood glucose levels compared to CT, glycemia levels observed in this study were all close to the concentration of 70 mg dL^{-1} which is accepted as normal glycemia for this species under rearing conditions (Gomes et al., 2003).

Blood Na^{+} concentration decreased and it was the only variable that did not return to CT levels after 72 h in recovery. Previous reports have described ion losses following anaesthesia in fish (Baldisserotto et al., 2008; Gressler et al., 2014). Ion losses by the gills and water influx can occur as a consequence of transitory osmoregulation disturbances and are frequently reflected in the osmolality, which did not occur in this study. Non-severe electrolyte alterations in fish also could be explained by normal ion diffusion in the gills, because a flux of ions of small magnitude could happen with many physiologic or environmental manipulations (Stoskopf, 1993).

In the face of mild changes on concentration of the ions K^{+} and Ca^{++} and more consistently Na^{+} after 2 h, coupled with a presumable transitory incapacity of controlling excessive water influx through the gills, it may have been possible that this scenario contributed to the onset of the oscillatory pattern observed in Hct and Hb after

2 h in recovery. The exact mechanism of action contributing to the reduction on Hct and Hb content is undetermined. Pseudoanemia may be caused by perturbations of fluid balance that lead to plasma expansion, thus causing apparent reductions of haemoglobin, red cell count and haematocrit, *i.e.*, haemodilution (Evans, 2009).

Unexpectedly, fish did not show significant alterations in blood pO_2 at any given sampling time. Environmental and/or physiological conditions that lower ventilation, such as exposure to hyperoxia or in this case anaesthetic induction, typically elicit a respiratory acidosis owing to CO_2 retention (Perry & Gilmour, 2006). Such a response was not observed in tambaqui of this study. A respiratory alkalosis characterized by depressed arterial pCO_2 and elevated arterial pH occurs when ventilation volume is increased in response to aquatic hypoxia even at the expense of disturbances of acid-base balance (Gilmour, 2001). Along with hypocapnia observed in this study, blood pH increased, which was consistent with respiratory alkalosis during the first 24h in recovery. Presumably, hyperventilation occurring early in recovery contributed to the transient respiratory alkalosis with enhanced CO_2 transfer before pCO_2 returned to similar levels of CT fish after 48h. Furthermore, the fact that those variables were negatively correlated is consistent with this assumption.

Blood HCO_3^- levels are increased during compensation of acidosis and decreased during compensation of alkalosis (Perry & Gilmour, 2006). The transient decrease in HCO_3^- levels observed immediately after recovery from anaesthesia (0 h) preceded the alkalosis observed at 2 h in recovery, however, HCO_3^- returned to CT levels at 2 h, which suggests a minor interference on HCO_3^- blood levels after anaesthesia.

Biological effects of plant extracts represent a promising line of study (Cunha et al., 2010; Silva, et al., 2012). Much of the information regarding the use of new anaesthetics still lack systematic evaluation of their impacts on behaviour, physiology and/or pathology in fish. Anaesthesia with jambu extract in this study proved to be efficient and elicited temporary physiological changes in blood mostly between 2 and 24h in recovery. It has been suggested that the alleged anaesthesia-induced stress may be a consequence of hypoxia/hypoventilation observed during anaesthesia rather than a direct effect of the drug (Rothwell et al., 2005). No mortalities were observed until 2 weeks after the experiments.

The extract of jambu proved to be an efficient anaesthetic for fish. The search for effective anaesthetics suitable for use in fish and also intended for human consumption without prolonged withdrawal period remains a priority in aquaculture. Along with the efficacy of a compound, as novel chemicals for immobilization of fish are discovered, evaluation of physiological effects and toxicity must lend credence to the use of the chemicals. It is imperative that the efficacy and safety of immobilization chemicals for use in fish are systematically, qualitatively, and quantitatively evaluated (Sladky et al., 2001).

In conclusion, the extract of jambu flowers, *S. acmella* is effective and safe for inducing slight sedation in tambaqui at 2 mg L^{-1} and deep anaesthesia at all concentrations tested in this study. Furthermore, among the concentrations tested in the present study, the use of 20 mg L^{-1} of this compound is suitable to induce fast anaesthesia (in less than 3 min) and promotes mild physiological changes in blood during recovery. Regarding human safety concerns, the fact that this plant has been widely and long used in gastronomy may facilitate its approval for use as an anaesthetic in fish destined for human consumption.

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Table 1: Anaesthesia and recovery stages in fish.

Stages	
Anaesthesia	
A1	Agitation
A2	Loss of equilibrium and erratic swimming
A3	Minimum opercular beating and loss of reaction to tail pinch stimulus (anaesthesia)
Recovery	
R1	Erratic swimming and recovery of equilibrium
R2	Regular opercular beating and normal swimming

Modified from Park et al., 2008.

Table 2. Anaesthesia induction and recovery times (in seconds ± SD) in juvenile Tambaqui, *Colossoma macropomum* exposed to five concentrations of jambu extract *Spilanthes acmella*.

Concentration (mg L^{-1})	*Induction (s)*		*Recovery (s)*		
	stage A1	*stage A2*	*stage A3*	*stage R1*	*stage R2*
5	199 ± 39^{a}	348 ± 72^{a}	459 ± 107^{a}	34 ± 17	271 ± 118^{ab}
10	104 ± 55^{b}	181 ± 48^{b}	269 ± 70^{b}	47 ± 30	281 ± 63^{ab}
15	82 ± 40^{bc}	120 ± 46^{c}	216 ± 63^{bc}	34 ± 27	233 ± 90^{b}
20	58 ± 29^{bc}	103 ± 32^{c}	173 ± 40^{c}	37 ± 22	300 ± 64^{ab}
25	48 ± 21^{c}	75 ± 17^{c}	144 ± 26^{c}	37 ± 28	338 ± 37^{a}
Equations	$y = 820.92x^{-0.88}$ $r^2 = 0.99$	$y = 1531.5x^{-0.925}$ $r^2 = 0.99$	$y = 1416.4x^{-0.706}$ $r^2 = 0.99$	-	$y = 0.4913x^2 - 11.671x + 324.82$ $r^2 = 0.74$

Different superscripts in the same column indicate statistical difference among concentrations after ANOVA and Tukey test ($n = 10$, $p<0.05$).

Table 3. Induction and recovery times (sec.) in tambaqui, *Colossoma macropomum* with different anaesthetics.

Anaesthetic	Recommended Concentrations[+] (mg L^{-1})	Induction time (s)	Recovery time	Authors
Benzocaine	100 – 150	103 – 163	275 – 532	Gomes et al. (2001)
Menthol	150	130	656	Façanha & Gomes (2005)
Eugenol	65	152	410	Roubach et al. (2005)

[+] Concentrations necessary to promote fast and deep anaesthesia (corresponding to stage A3).

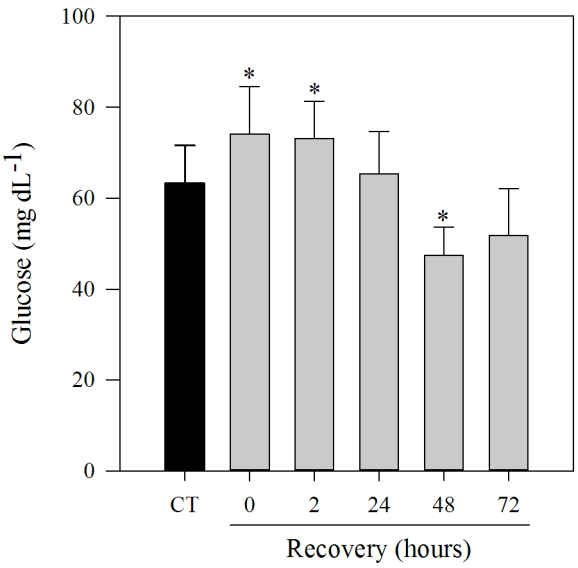

Fig. 1 - Glycemia in juvenile tambaqui *Colossoma macropomum* recovering from anaesthesia with extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}. CT corresponds to sham control fish. Five sampling times (0 - 72 h) were established in recovery. Values are means ± SD. Columns with an asterisk are significantly different from CT after ANOVA and Dunnet test ($n = 10$, $p<0.05$)

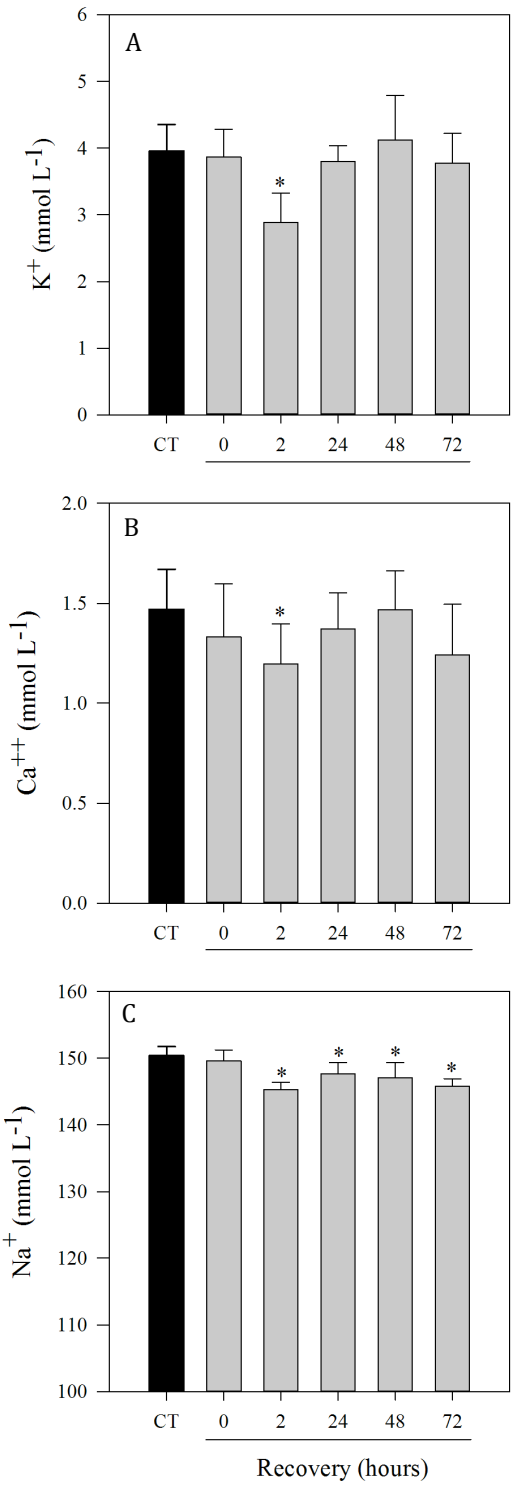

Fig. 2 - Whole blood ionic concentration in juvenile tambaqui *Colossoma macropomum* recovering from anaesthesia with extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}. CT corresponds to sham control fish. Five sampling times (0 - 72 h) were established in

recovery. Values are means ± SD. Columns with an asterisk are significantly different from CT after ANOVA and Dunnet test (n = 10, $p<0.05$).

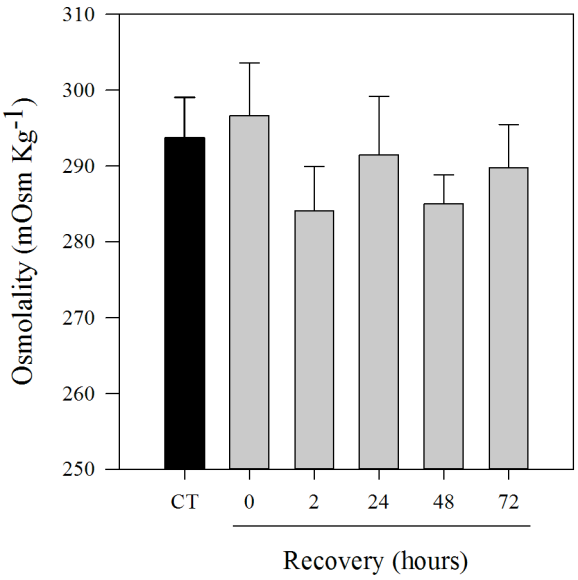

Fig. 3 - Whole blood osmolality in juvenile tambaqui in recovery after exposure to extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}. CT corresponds to sham control fish. Five sampling times (0 - 72 h) were established in recovery. Values are means ± SD. No significant differences were observed after ANOVA and Dunnet test (n = 10, $p<0.05$).

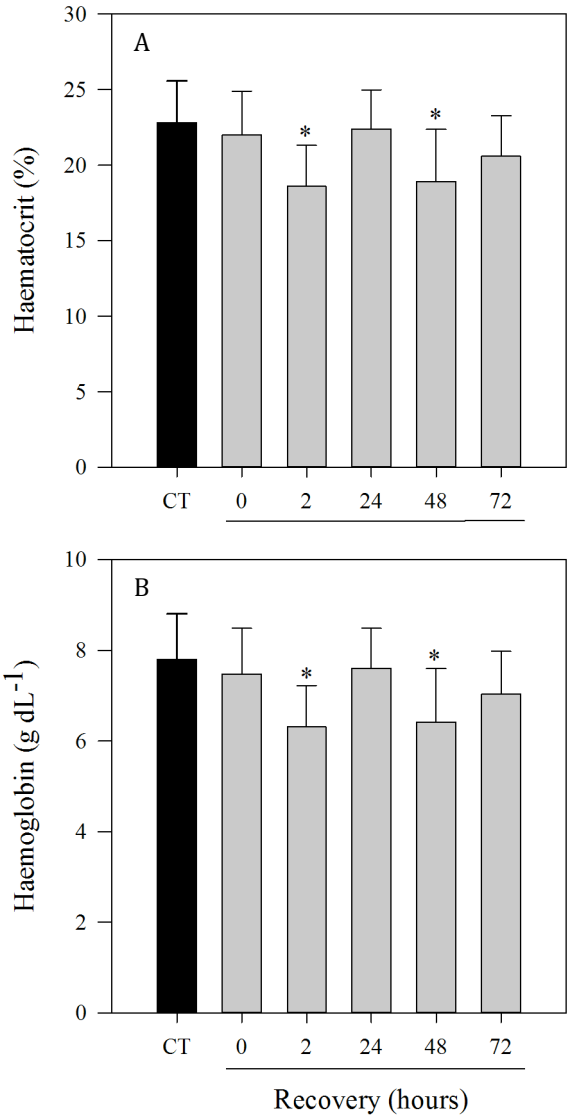

Fig. 4 - Haematological variables in juvenile tambaqui *Colossoma macropomum* recovering from anaesthesia with extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}. CT corresponds to sham control fish. Five sampling times (0 - 72 h) were established in recovery. Values are means ± SD. Columns with an asterisk are significantly different from CT after ANOVA and Dunnet test (n = 10, $p<0.05$).

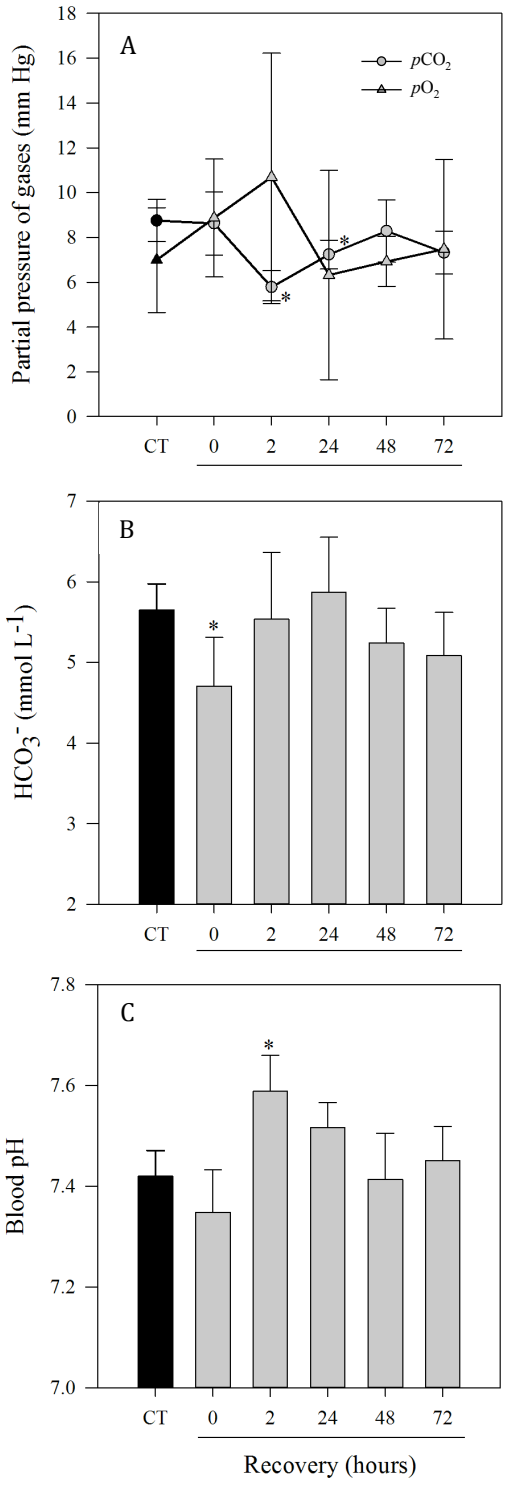

Fig. 5 - Blood partial pressure of gases (A), HCO_3^- (B) and pH (C) in juvenile tambaqui *Colossoma macropomum* recovering from anaesthesia with extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}. CT corresponds to sham control fish. Five sampling times (0 - 72

h) were established in recovery. Values are means ± SD. Columns with an asterisk are significantly different from CT after ANOVA and Dunnet test (n = 10, $p<0.05$).

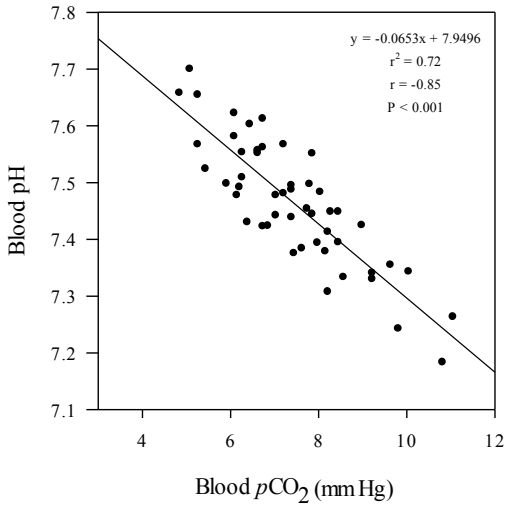

Fig. 6 - Relationship between blood pH and pCO_2 in juvenile tambaqui *Colossoma macropomum* in recovery from anaesthesia with extract of jambu, *Spilanthes acmella* at 20 mg L^{-1}.

CAPÍTULO 2

Anaesthesia and transport of juvenile tambaqui *Colossoma macropomum* with tricaine (TMS®): Implications on oxidative stress responses.

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Running title: Anaesthesia and transport of tambaqui with tricaine (TMS®)

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Abstract

The aim of this study was to evaluate the anaesthetic efficacy of tricaine (TMS®) in juvenile tambaqui, *Colossoma macropomum* as well as to investigate the effect of a lower sedation concentration on oxidative stress status of fish after transport in hyperoxia for 2, 6 and 10 h. For the verification of anaesthetic efficacy, juveniles were placed in aquaria containing six different concentrations of buffered tricaine (150, 180, 210, 240, 270 and 300 mg L^{-1}) and the times of anaesthetic induction and recovery were determined. Fish transported in hyperoxic conditions for 2, 6 and 10 h with or without anaesthetics were investigated for total antioxidant capacity against peroxyl radicals (ACAP), glutathione-S-transferase (GST) activity and lipoperoxidation levels (TBARS) in gills, brain and liver. TMS® induced deep anaesthesia in juvenile tambaqui, *C. macropomum* for all concentrations tested and the use of 240 mg L^{-1} of TMS® was capable of inducing fast and deep anaesthesia in less than 3 min. The use of a lower sedation concentration of 20 mg L^{-1} of TMS® did not imply clear advantages during transport for up to 10 h in terms of oxidative stress mitigation since responses between fish transported in the presence of this anaesthetic were generally undistinguishable compared to responses of fish transported in anaesthetic-free water, mainly in the case of lipoperoxidation levels.

Introduction

Tricaine methane-sulphonate (TMS®), $C_9H_{11}O_2N + CH_3SO_3H$, also known as MS-222 is currently the most used anaesthetic compound for fish worldwide. It is an isomer of benzocaine with an additional sulphonate radical, making it more soluble, but also more acidic, in solution (Congleton, 2006) and therefore requires the use of a buffering agent in order to obtain the desired pH and avoid metabolic acidemia in fish (Sneddon, 2012).

Tricaine is the only US Food and Drug Administration approved anaesthetic for use on fish destined for human consumption. It is also registered for veterinary use in the UK, Canada, Italy, Spain and Norway (Sneddon, 2012; Popovic et al., 2012). TMS® is routinely used both for non-invasive and surgical procedures in fish, it is also recommended as the first step of euthanasia for the majority of laboratory fish

(Readman et al., 2013).

Transport of fish in closed systems is also done with plastic bags, usually filled 1/3 with water and 2/3 with pure oxygen. As a consequence there is a rapid increase of dissolved oxygen levels in the water at the beginning of transportation (Golombieski et al., 2003; Gomes et al., 1999).

Hyperoxia is a state that promotes generation of elevated levels of Reactive Oxygen Species (ROS) and the production of ROS is balanced by enzymatic and nonenzymatic (low-molecular weight compounds) antioxidant systems (Halliwel & Gutteridge, 1999). The perturbation of the pro-oxidant/antioxidant balance can lead to increased oxidative damage of macromolecules, a phenomenon known as oxidative stress (Bisbal et al., 2010). Exposure to high concentrations of dissolved oxygen in the water induced oxidative stress in different fish species such as goldfish *Carassius auratus* (Lushchak et al., 2005), Atlantic salmon *Salmo salar* (Olsvik et al., 2005) and Senegal sole *Solea senegalensis* (Salas-Leiton et al., 2009). Therefore, it is clear that the increased level of external oxygen is an inducer of oxidative stress in aquatic animals.

The use of anaesthetics during transport of juvenile fish is recommended to reduce stress (Ross & Ross, 2008). However, it has been reported that the application of anaesthetic concentrations of TMS® (at 100 mg L^{-1}) slightly altered internal organs and tissues of rainbow trout, resulting in increased production of ROS, leading to oxidative damage to lipids and proteins with inhibition of antioxidant capacities (Velisek et al., 2011). Conversely, it has been hypothesized herein if a lower concentration of TMS® added to the transport water of juvenile fish is capable of attenuating oxidative stress responses under hyperoxia.

Tambaqui, *Colossoma macropomum*, is an important Amazon species in the north of Latin America (Araújo-lima and Goulding, 1997; Sevilla and Günther, 2000) This is the leading native aquaculture species in Brazil, with production above 111 metric tons per year (MPA, 2011). This fish presents good growth rates and feed conversion (Merola & Cantelmo, 1987). To date, there are no reports about sedative and/or anaesthetic activity of TMS® in tambaqui. Furthermore, only a few reports (Azambuja et al., 2011; Salbego et al., 2014; Zeppenfeld et al., 2014) on the impacts of sedation coupled with transportation in hyperoxic conditions on oxidative stress responses in fish are available.

The aim of this study was to evaluate the anaesthetic efficacy of TMS® for juvenile tambaqui, as well as to investigate the effect of sedation with this compound on oxidative stress responses post-transport.

Materials and methods

Anaesthetic

Tricaine (TMS®) (Sigma Chemical E10521 - St Louis, MO, USA) used in experiments was solubilized in deionized water and buffered with sodium bicarbonate, using a ratio of 1:1 (sodium bicarbonate:tricaine methanesulfonate powder), providing a solution with a final TMS® concentration of 10 mg mL^{-1} (pH 7.0) from which aliquots were taken and used in the water of the trials. Buffered TMS® was only prepared on the day of the experiments and kept in ambar bottle until its use.

Animals

Juvenile tambaqui, irrespective of sex, were obtained from a fish farm in the State of Amazonas, northern Brazil. Animals were housed indoors in recirculating aquaculture systems. For the 15-day acclimation period, specimens were placed in 250 L tanks, stocking density ~ 5 g L^{-1}, with approximately 200 L of fresh water that was constantly aerated by means of 20 W pumps and air stones. Fish were fed twice a day with a commercial diet for omnivorous fish (28% crude protein). The quantity of food offered corresponded to 2% of their biomass per day during the acclimation period. Thirty minutes after the feeding, the faeces and food remains were siphoned out and new water was added to the tank to make up the volume.

This study was approved by the Ethics Committee on Animal Experimentation of the Universidade Federal do Rio Grande - FURG.

Water Quality

In the acclimation tanks dissolved oxygen (DO) (6.40 ± 0.37 mg L^{-1}) and temperature (26.0 ± 1.69 °C) were measured using an oxygen meter (Yellow Springs Instruments, USA); pH (6.8 ± 0.1) was determined with a pH meter (Five Easy FE20, Switzerland). Total ammonia nitrogen (TAN) (0.80 ± 0.1 mg L^{-1} $NH_4^+ + NH_3^-$ - N) was quantified according to Unesco (1983) and nitrite (0.03 ± 0.01 mg L^{-1}) was determined according to Bendschneider & Robinson (1952). Total alkalinity (60.7 ± 0.9 mg $CaCO_3$ L^{-1}) was evaluated by titration in accordance with Eaton et al. (2005) guidelines.

Experiment 1: Induction and recovery stages of anaesthesia with TMS®

After the acclimation period, juveniles (15 ± 3 g; 10 ± 1 cm, total length) were fasted for a period of 24 h prior to the experiments. The fish were netted and transferred individually to the anaesthetic induction tank (30L) with 10 L of the same water from the acclimation tanks. Tambaqui were individually exposed (n=10, each fish being a replicate) to six TMS® concentrations (150, 180, 210, 240, 270 and 300 mg L^{-1}) diluted in the water of the induction tank. A fresh anaesthetic solution was prepared after each five fish were individually tested, in order to assure good water quality and to accurately guarantee the anaesthetic concentration to be used in the water. The induction and recovery time were measured under the same experimental conditions using a digital stopwatch.

Changes in behavioural of the anaesthetized fish were evaluated and stages were characterized as described by Park et al. (2008) with modifications (Table 1).

Fish behaviour (stages) was monitored for each concentration. The total length and weight of the individual fish were measured and registered after anaesthesia. The recovery stage was recorded after transferring the fish to anaesthetic-free aerated water in the recovery tank (30 L). Fish were declared recovered when their movement and equilibrium were completely re-established. Following the recovery stage, juveniles were transferred to holding tanks and were observed daily for a 4-day period for any abnormal behaviour or mortality.

Experiment 2: Transport and evaluation of oxidative stress status.

Water quality parameters: temperature, alkalinity, dissolved oxygen, pH and total ammonia were determined at the beginning (0 h) and at the end of each transport period.

Tambaqui juveniles (66 ± 15 g and 16 ± 1 cm, total length) were used for this experiment. According to experiment 1 and in order to establish a suitable sedation concentration for transports, pre-tests were performed and some specimens were exposed for 10 min to TMS® baths in concentrations ranging from 5 to 10% of the minimum anaesthetic concentration (240 mg L^{-1} in experiment 1) that was capable of inducing anaesthesia in less than 3 min. At the concentration of 20 mg L^{-1} of TMS®, which corresponded to approximately 8% of the aforementioned anaesthetic concentration, sedation was achieved, clinically characterized by lethargy and reduced response to external stimuli. This condition is considered appropriate for non-invasive procedures such as transport when only sedation is necessary (Sneddon, 2012).

Juveniles were weighed and placed in plastic bags (30 L) with 5 L of water that were inflated with pure oxygen and tied with rubber bands. The load density was set at approximately 160 g L^{-1} (12 specimens per bag and bags were in triplicate per group), which corresponds to the density used in commercialization of this species in the Amazon region. Fish were transported on paved road for two, six and ten hours with or without TMS® at 20 mg L^{-1}

At the end of each period (2, 6 and 10 h), six specimens per group (2 per bag) were taken and killed by spinal sectioning. Gills, liver and brain were removed and tissues were weighed and immediately stocked in a freezer at − 80 °C for subsequent analysis of antioxidant status and lipoperoxidation (LPO) levels in organs. Tissues of non-transported fish (six individuals) from the acclimation tanks in normoxic conditions and not exposed to TMS® were used to provide initial reference values of ACAP, GST and LPO levels.

Oxidative stress parameters

Briefly, tissues were homogenized at a rate of 1:5 (w v^{-1}) in buffer (Tris-HCl – 100 mM; EDTA – 2 mM; and $MgCl_2.6H_2O$ – 5mM) (Da Rocha et al., 2009). The supernatants resulting from the centrifugation of the homogenates (10,000 x*g*, 20 minutes, 4ºC) were used for all analyses. After homogenization, total protein content was determined through Biuret method. Thereafter, samples of gill and liver were diluted with buffer to 2.0 mg protein mL^{-1} and brain to 0.5 mg protein mL^{-1} for posterior analysis of total antioxidant capacity against peroxyl radicals.

Total antioxidant capacity against peroxyl radicals was determined according to the method described by Amado et al. (2009). This method consists of finding the antioxidant capacity of tissues using a fluorescent substrate (2′,7′ dichlorofluorescein diacetate - H_2DCF-DA) and the production of peroxyl radicals by thermal decomposition of ABAP (2,2′-azobis 2 methylpropionamidine dihydrochloride). The fluorescence was determined through a microplate reader (Victor 2, Perkin Elmer), at 37 ºC (excitation: 485 nm; emission: 530 nm) with readings at every 5 minutes, during 30 minutes. The results were expressed as a relative area (the difference between the area with and without ABAP divided by the area without ABAP). Using this methodology, a lower relative area means higher antioxidant capacity.

The lipoperoxidation of all tissues was measured using the methodology described by Oakes & Van Der Kraak (2003). To determine the thiobarbituric acid reactive substances (TBARS) by the quantification of MDA (malondialdehyde), 20 µL of BHT solution (67µM), 150 µL 20% acetic acid solution, 150 µL 0.8 % TBA solution, 50 µL Milli-Q H_2O, and 20 µL of 8.1% SDS were added to 30 µL of each sample homogenate before being heated at 95 ºC with water bath for 30 minutes. Thereafter, 100 µL of Milli-Q H_2O and 500 µL of n-butanol were added to the final solution. The remaining supernatant of centrifugation (3000 rpm, 10 minutes, 15 ºC) was used to determine the fluorescence (excitation: 520 nm; emission: 580 nm) and the results were expressed as nmol TMP mg $protein^{-1}$, where TMP stands for the standard Tetramethoxypropane (ACROS Organics).

Determination of glutathione-S-transferase (GST) activity was performed according to Habig et al. (1974). Potassium phosphate buffer (K_2HPO_4 – 0.05 M and

Milli-Q H_2O; pH 7.0) was used as reaction medium (previously heated at 25 ºC with water bath) and then substrate 1-chloro-2,4-dinitrobenzene (CDNB) (50 mM) and reduced glutathione (GSH, 25 mM) were added. CDNB and GSH were added together with 10 µL of sample homogenate in transparent 96 wells microplate and read at 340 nm using microplate reader (Victor 2, Perkin Elmer). The results were expressed as nmol of CNDB-GSH conjugate mg protein^{-1} min^{-1}.

Statistical analysis

All data are presented as mean ± SD. To verify the normality and homogeneity of variances, data were submitted to Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA and Tukey tests were used for data of anaesthesia induction and recovery times (experiment 1). Two-way ANOVA (with transport length and anaesthetic being used as factors) and Tukey tests were performed to check differences between water quality variables and oxidative stress parameters in experiment 2. Water quality parameters before and after transport were submitted to repeated measures Anova and Dunnet test. Since data could not be homogenized, the non-parametric Kruskal–Wallis test was used (Zar, 1996) to test the results obtained for ACAP, GST activity and TBARS in liver. Trends of the anaesthesia stages and recovery were fitted using non-linear response models with measured times to reach stages being the response variables and concentrations of TMS® the predictor variable. In all cases the minimum significance level was set at $p<0.05$.

Results

Fish were monitored after each trial and no mortalities were observed until 4 days after the trials in experiment 1 and among animals that were not used for tissue samplings and returned to the original tanks after experiment 2.

Experiment 1: Induction and recovery stages of anaesthesia with TMS®

Baths with TMS® did not promote agitation behaviour (A1) at any concentration tested. Increasing concentrations of TMS® gradually decreased the time to show loss of equilibrium (A2) and deep anaesthesia with loss of reaction to tail pinch stimulus (A3) (Table 3). All concentrations tested promoted deep anaesthesia (A3) within the stipulated observation time limit of 30 min, and concentrations of 240 mg L^{-1} and forward were effective in inducing anaesthesia in less than 3 min (180 s), hereafter referred to as fast anaesthesia.

Based on visual assessment fish were acknowledged fully recovered after re-establishing of equilibrium and normal swimming for all concentrations tested. Fish exposed to 150 mg L^{-1} recovered faster ($p<0.05$) from anaesthesia compared to all other concentrations used, except when compared to the recovery time of animals exposed to 300 mg L^{-1} ($p>0.05$). Non-linear patterns were observed for induction and recovery times over increasing concentrations of TMS®, which can be fairly estimated by the models proposed herein (see equations in Table 3).

Experiment 2: Transport and evaluation of oxidative stress status

Water quality

Water quality parameters were not significantly different ($p>0.05$) over time for transports with or without TMS® and no differences were observed between groups with TMS® and controls transported without TMS® within the same time of transport as well. The only exception were final dissolved oxygen levels, which significantly decreased ($p<0.05$) after 6 h of transport and forward regardless of the presence of TMS®. Temperature and pH were significantly reduced ($p<0.05$) after transport, regardless of transport duration or the presence of TMS® compared to water before transport. No significant alterations in water alkalinity were observed after transport compared to water before transport, except for transports after 6 h without anaesthetics in which this variable was increased ($p<0.05$). Total ammonia concentration was

significantly increased ($p<0.05$) after transports for all treatments compared to total ammonia levels in the water before transport (Table 2).

Gills

No differences ($p>0.05$) were observed in ACAP between groups with or without TMS® within the same transport time or over time. All transported groups showed the same ($p>0.05$) ACAP of non-transported fish (Fig 1A).

GST activity was not different ($p>0.05$) within each transport time for fish transported with or without TMS®. Non-transported fish showed higher GST activity ($p<0.05$) compared to all transported groups (Fig 1B).

TBARS concentration was not significantly changed ($p>0.05$) in gills regardless of transport time or the presence of TMS® (Fig 1F). TBARS concentration was also unchanged ($p>0.05$) compared to non-transported fish, except for an increase ($p<0.05$) observed in fish transported for 2 h without TMS®, nevertheless, not being different ($p>0.05$) from fish transported with TMS® for the same length of time (Fig 1C).

Brain

Fish transported for 6 and 10 h in the presence of TMS® showed decreased ACAP ($p<0.05$) compared to fish transported for 2 h in the presence of the anaesthetic (Fig 1D). Moreover, fish transported for 6 h with TMS® showed decreased ACAP compared to control fish transported for the same time. Despite of these findings, no differences ($p>0.05$) were observed between ACAP in any transported group compared to non-transported fish (Fig 1D).

Fish transported with TMS® showed higher GST activity ($p<0.05$) after 2 h transport compared to controls within the same transport time (Fig 1E). However, no significant changes ($p>0.05$) were observed in controls over time and no differences were observed ($p>0.05$) between transported groups and non-transported fish (Fig 1E).

Although significant reductions ($p<0.05$) in TBARS were observed after transport for 6 and 10 h in the presence of TMS® compared to fish transported for 2 h with the anaesthetic, lipoperoxidation levels in brain were not significantly different

(p>0.05) within each transport time or over time in control fish. Furthermore, no changes (p>0.05) were observed in TBARS for all transported groups compared to reference values of non-transported fish (Fig 1F).

Liver

Changes in antioxidant status occurred only between transported *vs.* non-transported group in which lower GST activity (p<0.05) was observed in the liver of animals transported for 6 and 10 h in controls and in fish transported in the presence of TMS®, respectively, compared to GST activity of non-transported fish (Fig 1H). TBARS levels increased (p<0.05) in control fish transported for 2 h and in fish transported with TMS® for 6 h compared to values of non-transported fish (Fig 1I). Notwithstanding, ACAP, GST activity and TBARS in liver were unchanged among transported fish irrespective of the presence of TMS® or transport time (p>0.05) (Fig 1 G, H and I).

Discussion

TMS® efficacy

The efficacy of TMS® was comproved for juvenile tambaqui *C. macropomum*. The onset of individual stages of anaesthesia and recovery times depended on the anaesthetic concentrations used. In general, induction time decreased and recovery time increased with increasing concentrations.

Juvenile tambaqui anaesthetized with TMS® did not show suggestive signs of aversion (A1) such as rapid flaring of the opercula, 'coughing', surfacing or frantic swimming. Adverse reactions have been reported for initial stages of anaesthesia in fish (APC, 2009; Readman et al., 2013) and some reactions are described as 'coordinated excitatory behaviour with increased respiratory rate' or hyperactivity, followed by 'violent thrashing and jumping' before eventually loosing equilibrium (Lewbart, 1998; Grush et al., 2004; Mylonas et al., 2005; Ross & Ross, 2008). Although concerns have been raised that TMS® can cause aversive reaction and irritation of tissues in fish

(Williams et al., 2009; Reed & Jennings, 2011; Readman et al., 2013), tambaqui did not show distressed behaviour when exposed to the drug, instead, had a smooth transition to stage A2 for all concentrations tested.

Stage A2 is a transitory phase characterized by loss of the righting reflex and ataxia (see Table 1 for the description of induction stages) (Park et al., 2008) that takes place immediately before deep anaesthesia is achieved. Time to loss of equilibrium and erratic swimming was significantly anticipated with increasing concentrations of TMS® and this trend can be clearly predicted by exponential regression.

Fast anaesthesia was reached in 2.7 min (162 s), and time range between 1.8 and 2.8 min (108 and 168 s, respectively) in recovery were in compliance with the recommended maximum times of 3 (180 s) and 5 min (300 s) for induction and recovery, respectively (Bell, 1987, Iwama & Ackerman, 1994; Ross & Ross, 2008)

Effective anaesthetic concentrations of TMS® may vary between species as previously reported, *e.g.*, in Atlantic salmon (*Salmo salar*) it is administered at 65 mg L^{-1} (Kiessling et al., 2009), whereas in halibut (*Hippoglossus hippoglossus*) TMS® is given at 80 mg L^{-1} (Zahl et al., 2011) and in cod (*Gadus morhua*) it is effective at 60 mg L^{-1} (Zhal et al., 2009). The lowest concentration of TMS® (150 mg L^{-1}) tested herein capable of inducing deep anaesthesia in tambaqui was higher to at least 1.8-fold compared to those aforementioned anaesthetic concentrations, therefore this species seems to be relatively less sensitive to this anaesthetic and requires higher anaesthetic concentrations, mainly in the case of fast anaesthesia.

Other compounds such as benzocaine, menthol and eugenol have been tested for tambaqui with recommended concentrations of 100-150, 150 and 65 mg L^{-1} respectively (Gomes et al., 2001; Façanha & Gomes, 2005; Roubach et al., 2005). Those were regarded as appropriate concentrations to promote fast anaesthesia in less than 3 min and are lower effective concentrations compared to the ones used in the present study. Nevertheless, menthol and eugenol allowed recovery only after 5 min, thereby exceeding the maximum time recommended for recovery from anaesthesia in fish (Bell, 1987; Iwama & Ackerman, 1994; Ross & Ross, 2008). Nevertheless, great care should be used when comparing stages of anaesthesia and efficacy of a given compound for fish, since environmental and intraspecific conditions, pre-dilution of the compounds, health conditions and weight of animals are some of the variables that are likely to

influence anaesthesia outcome in fish (Burka et al., 1997; Ross & Ross 2008; Sneddon, 2012).

Oxidative Stress Response

With regards to water deterioration post-transport, it seems that decreases in water DO levels over time were not indirectly prevented by the addition of TMS® which was expected to occur through reduction of metabolism. Although oxygen availability was decreased in transports over time independently of the presence of TMS®, values were still within tolerance limits for juvenile tambaqui (Saint-Paul, 1988) at the end of all transports. Although total ammonia levels were increased at the end of the transports, tambaqui is a highly tolerant to ammonia toxicity (Ismiño-Orbe, 1997). Due to the slightly acidotic waters and decreased temperatures observed at the end of all treatments, a very low toxic fraction of ± 0.1% non-ionized ammonia was present and can be estimated as a function of temperature and pH of the water according to published tables (Boyd, 1982).

Total antioxidant capacity in tissues of juvenile tambaqui seemed not to be disturbed irrespective of transport time or the presence of TMS®. In the face of pro-oxidant conditions, enzymatic and nonenzymatic antioxidant systems usually play an important role in order to protect tissues against oxidative damage. In turn, development of oxidative stress is evidenced by alterations in levels of oxidative damage markers (Storey, 1996).

The conjugation of several xenobiotics with glutathione (GSH) is mediated by GST enzyme as part of the detoxification process (Habig et al., 1974) and also for the protection of cells against residual products originated from the peroxidation of tissues (Van der Oost et al., 2003). In general GST activity was not altered in tissues of transported fish, regardless of the presence of TMS®, except for a higher GST activity in brain of fish transported for 2 h with TMS® compared to fish transported without anaesthetic for the same time.

In gills and liver it was not clear the advantages of using TMS® since LPO levels between transported fish were undistinguishable both within and over transport time. Although some transported fish showed increased LPO levels in those tissues

irrespective of the presence of TMS® compared to levels of non-transported fish in normoxia, higher LPO levels in those cases were rather expected, since resting fish under normoxic conditions are likely to show lower levels of LPO. Those results corroborate previous findings of more severe LPO levels for fish exposed to hyperoxia *vs.* fish maintained in normoxia (Lushchak et al., 2005).

Polyunsaturated fatty acids are abundant in brain tissue, therefore one can expect that this organ is prone to oxidative damage caused by hyperoxic environments (Azambuja et al., 2011). The variations observed in antioxidant responses in brain (Fig 1D and E) of tambaqui were presumably adaptive responses to cope with the challenge posed by the transport in hyperoxic water, which in turn was not enough to cause damage through LPO in this organ compared to non-transported fish. Unchanged GST activity and increased TBARS levels in brain have been reported for goldfish exposed to short-term (3 to 6 hours) hyperoxic conditions (Lushchak et al., 2005), however, differently from those findings, brain of tambaqui exposed to transports in hyperoxia showed increased GST activity after 2 h of transport in the presence of TMS® whereas LPO levels of fish submitted to hyperoxic transport waters were unchanged ($p>0.05$) compared to TBARS of non-transported fish maintained in normoxia.

With regards to gill responses, our results resemble those of silver catfish *Rhamdia quelen* transported in hyperoxia for 5h with the anaesthetic essential oil of *Lippia alba* in which no significant changes in TBARS or GST activity in gills were observed when compared to individuals transported without anaesthetic for the same length of time (Azambuja et al., 2011).

It has been reported that anaesthesia exposure for 10 min with MS-222 at the concentration of 100 mg L^{-1} increased the concentration of TBARS in brain, muscle, liver and intestine of rainbow trout *Oncorhynchus mykiss* after 24h in recovery compared to non-anaesthetized fish, whereas analyses of these tissues immediately after the 10 min exposure did not elicit changes in LPO levels (Velisek et al., 2011). Therefore, there is a potential for the anaesthetic itself or the anaesthetic-induced short-term hypoxia followed by reoxygenation to induce an oxidative insult that could be detected later in recovery. Oxidative stress responses were not investigated after deep anaesthesia with TMS® in this study, therefore the existence of a direct or indirect

anaesthetic-driven pro-oxidant condition in tambaqui during recovery after deep anaesthesia with TMS® is unknown.

All concentrations of TMS® tested herein were effective to promote calm (absence of stage A1) and deep anaesthesia (stage A3) in *C. macropomum* juveniles and concentrations of 240 mg L^{-1} and forward are suitable concentrations to promote safe and fast anaesthesia with uneventful recovery in this species. With respect to oxidative stress responses, although LPO levels were significantly increased in gills and liver of some groups transported with or without TMS® compared to LPO levels of non-transported fish, sedation with TMS® at the concentration of 20 mg L^{-1} neither intensified nor ameliorated oxidative stress in fish transported for up to 10 h when compared to control fish transported without TMS®.

In conclusion, TMS® is an effective anaesthetic for juvenile tambaqui *C. macropomum* and the concentration of 240 mg L^{-1} is recommended to promote fast and deep anaesthesia in this species. No clear advantages were obtained for the attenuation of oxidative stress responses with sedation concentrations of 20 mg L^{-1} added to the water for up to 10 h of transport since oxidative stress indicators were generally undistinguishable compared to responses of fish transported in anaesthetic-free water, mainly in the case of lipoperoxidation levels. Further studies are encouraged in order to follow oxidative stress responses of fish for longer periods of time during recovery after transport.

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Figure captions

Figure 1. Antioxidant status (ACAP and GST activity) and lipoperoxidation levels (TBARS) in gills (A, B and C), brain (D, E and F) and liver (G, H and I) of juvenile tambaqui, *Colossoma macropomum* submitted to different transport times (2, 6 and 10 h) with and without TMS® (MS-222). Values are expressed as means ± SD. When present, different lowercase letters indicate significant differences among transport time; * indicates significantly different from control within the same transport time; Means represented by open markers are significantly different from non-transported fish kept in normoxic conditions ($p<0.05$), n=6.

Table 1. Anaesthesia and recovery stages in fish.

Stages	Behavioural characteristics
Anaesthesia	
A1	Agitation
A2	Loss of equilibrium and erratic swimming
A3	Loss of responsiveness to mechanical stimuli and reduced opercular movement (deep anaesthesia)
Recovery	Normal opercular movement, re-establishment of equilibrium and normal swimming

According to Park et al. (2008) with modifications.

Table 2 - Water quality parameters (Mean ± SD) for juvenile tambaqui, *Colossoma macropomum* transported with or without TMS® (MS-222) for different times.

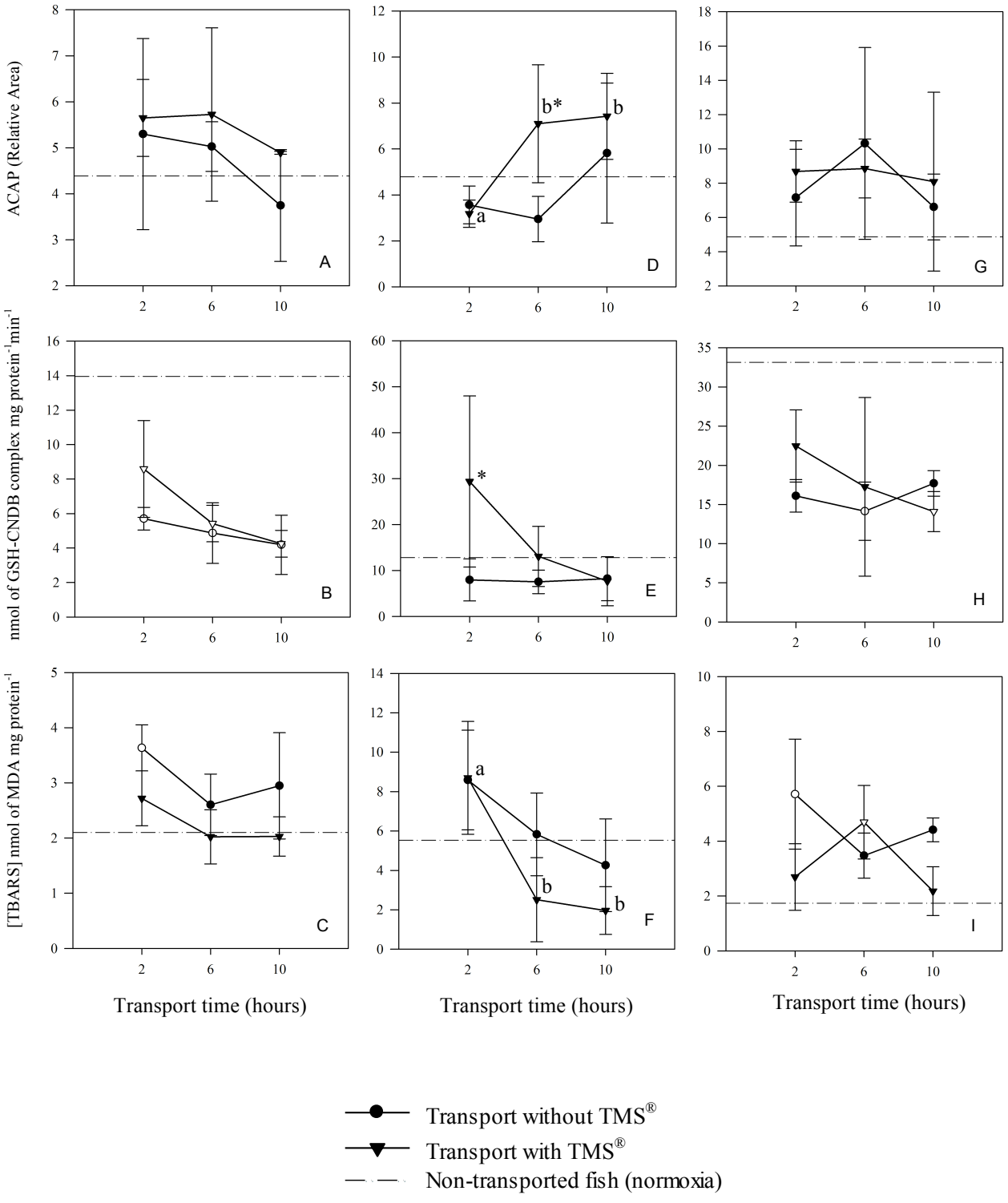
			Without TMS®	With TMS®
pH	BT		7.4 ± 0.02	
	AT	2 h	6.8 ± 0.06*	6.8 ± 0.04*
		6 h	6.4 ± 0.01*	6.5 ± 0.13*
		10 h	6.4 ± 0.02*	6.4 ± 0.07*
Alkalinity (mg $CaCO_3$ L^{-1})	BT		63.0±2.8	
	AT	2 h	65.0 ± 1.0	68.0 ± 2.8
		6 h	78.0 ± 2.5*	67.0 ± 7.6
		10 h	73.0 ± 2.5	63.0 ± 12.5
T (ºC)	BT		26.8 ± 0.3	
	AT	2 h	24.8 ± 0.1*	23.7 ± 0.2*
		6 h	24.2 ± 0.2*	24.4 ± 0.3*
		10 h	24.1 ± 0.1*	24.2 ± 0.1*
DO (mg L^{-1})	BT		8.4 ± 0.15	
	AT	2 h	21.1 ± 2.5a*	16.5 ± 3.0a*
		6 h	3.5 ± 0.6b*	8.9 ± 2.7b
		10 h	4.5 ± 1.8b*	3.5 ± 1.7b*
Total ammonia (mg L^{-1})	BT		0.7±0.1	
	AT	2 h	4.8 ± 0.9*	5.2 ± 0.9*
		6 h	6.5 ± 0.4*	6.4 ± 0.5*
		10 h	7.6 ± 0.9*	6.5 ± 0.7*

When present, different lower case letters in columns indicate statistical differences between times of transport. Treatments with or without TMS® did not show significant differences between water quality variables within the same time of transport. Data were submitted to two-way ANOVA and Tukey test at 5% of probability. * Indicates differences between water parameters before transport (BT) and after transport (AT) by repeated measures ANOVA and Dunnet test at 5% of probability.

Table 3. Induction and recovery time from tricaine methanesulphonate (TMS®) anaesthesia in juvenile Tambaqui, *Colossoma macropomum*. Time for stage transition is cummulative and is shown in seconds (s) ± SD.

Concentration (mg L^{-1})	Induction (s)			Recovery (s)
	stage A1	*stage A2*	*stage A3*	
150	-	198 ± 42 ^{a}	518 ± 78 ^{a}	108 ± 33 ^{b}
180	-	160 ± 50 ^{ab}	302 ± 54 ^{b}	168 ± 24 ^{a}
210	-	120 ± 32 ^{bc}	217 ± 76 ^{c}	175 ± 32 ^{a}
240	-	104 ± 29 ^{cd}	162 ± 48 ^{cd}	166 ± 45 ^{a}
270	-	73 ± 15 ^{d}	108 ± 24 ^{de}	175 ± 39 ^{a}
300	-	63 ± 16 ^{d}	72 ± 14 ^{e}	149 ± 42 ^{ab}
Equations	-	$y = 645.82e^{-0.008x}$ $r^2 = 0.98$	$y = 3180.2e^{-0.013x}$ $r^2 = 0.99$	$y = -0.0083x2 + 3.9447x - 288.11$ $r^2 = 0.83$

Stages are characterized according to Park et al (2008) with modifications. When present, different superscripts in columns indicate significant differences among concentrations by Tukey test ($p<0.05$, $n=10$).


ACAP (Relative Area)
nmol of GSH-CNDB complex mg protein-1min-1
[TBARS] nmol of MDA mg protein-1
A
B
C
D
E
F
G
H
I
b*
b
a
*
a
b
b
Transport time (hours)
Transport time (hours)
Transport time (hours)
Transport without TMS®
Transport with TMS®
Non-transported fish (normoxia)

CAPÍTULO 3

Anaesthetic efficacy and antioxidant properties of extractives of *Nectandra grandiflora* and *Spilanthes acmella* on juvenile tambaqui *Colossoma macropomum* submitted to transport

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Running title: Anaesthetic and antioxidant properties of new plant extractives for juvenile tambaqui

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Abstract

The aim of this study was to evaluate the anaesthetic activities of the extractives of *Nectandra grandiflora* and *Spilanthes acmella* in juvenile tambaqui fish, *Colossoma macropomum* as well as to investigate their effects on oxidative stress status of fish submitted to transport in hyperoxia. For the verification of anaesthetic activity, fish were placed in aquaria containing five different concentrations of essential oil (EO) of *N. grandiflora* (25; 50; 100; 200; and 300 μL L^{-1}) or waxy extract of *S. acmella* (5; 10; 15; 20; and 25 mg L^{-1}), and the times of anaesthetic induction and recovery were determined by dose-response trials. Fish transported inside plastic bags in hyperoxic conditions for 2, 6 and 10 h with or without anaesthetics were investigated for the occurrence of oxidative stress through the assessment of total antioxidant capacity against peroxyl radicals (ACAP), glutathione S-transferase (GST) activity and lipoperoxidation levels (TBARS) in muscle, gills, liver and brain. Extract of *S. acmella* and EO of *N. grandiflora* were effective and safe for inducing deep anaesthesia in juvenile *C. macropomum*. Moreover, the use of 10 mg L^{-1} of extract of *S. acmella* was suitable to induce fast anaesthesia (in less than 3 min). Antioxidant properties within these extractives enhanced protection against oxidative damage in organs of juvenile tambaqui. Sedation concentrations at 1 mg L^{-1} and 30 μL L^{-1} of the extractives of *S. acmella* and *N. grandiflora* respectively, are recommended for the transport of this species since those compounds reduced lipoperoxidation levels mainly in muscle and gills.

Keywords: Anaesthesia, hyperoxia, antioxidants, essential oil, fish, sedation, oxidative stress.

1. Introduction

Anaesthesia and sedation protocols are often used in aquaculture to minimize stress or physical damage caused by handling, sorting, tagging, artificial reproduction procedures and veterinary medicine practices (Coyle *et al.*, 2004; Ross and Ross, 2008; Roubach *et al.*, 2005). The characteristics of a given compound regarded as a good anaesthetic relies on its availability, cost, physical properties and safety during manipulation (Cho and Heath, 2000; Treves-Brown, 2000).

In order to mitigate the adverse effects of stress, several compounds are used as fish anaesthetics, being the most frequent tricaine methane-sulphonate (MS-222), quinaldine and 2-phenoxy-ethanol. Some of them can be toxic, associated with undesirable side effects, and expensive (Readman *et al.*, 2013; Roubach *et al.*, 2001; Svoboda and Kolarova, 1999). Although not regulated for use as an anaesthetic for fish, benzocaine has been recommended as an efficient compound for juvenile tambaqui, *C. macropomum* (Gomes *et al.*, 2001) and is frequently used in Brazil.

Since plants may be abundant and rich in chemicals that promote anaesthetic/analgesic activities, plant extractives such as waxes and essential oils (EO) (*e.g.* obtained by methodologies of supercritical fluid extraction technology with the use of CO_2 and ethanolic extraction, respectively) are a promising alternative to traditional synthetic drugs. Essential oils of *Lippia alba*, *Ocimum gratissimum* and *Aloysia triphylla* have been investigated for their anaesthetic efficacy in aquatic organisms (Azambuja *et al.*, 2011; Benovit *et al.*, 2012; Cunha *et al.*, 2010; Parodi *et al.*, 2012; Silva *et al.*, 2012). Menthol and eugenol have also been reported as good anaesthetics for fish (Façanha and Gomes, 2005; Roubach *et al.*, 2005).

The genus *Nectandra* (Lauraceae) includes more than 100 recognized species of canopy trees mainly distributed from Amazon to Southern Brazil, Argentina, Paraguay, and Uruguay (Alves and Sartori, 2009). This group of plants has been used in folk medicine as anti-rheumatic, analgesic (Santos Filho and Gilbert, 1975), sedative (Alves *et al.*, 2008), anti-inflammatory (da Silva *et al.*, 2004) and as antioxidant agents (Garcez *et al.*, 2009; Tondolo *et al*., 2013). *Nectandra grandiflora* Nees, is popularly known as "canela-amarela" and previous work has evidenced the presence of alkaloids with antitumoral activity in its bark extract (Moreno *et al.*, 1993). Antioxidant properties have also been described when using crude extracts from the leaves (Ribeiro *et al.*, 2005).

The genus *Spilanthes* is comprised of approximately 60 species widely distributed over tropical and subtropical regions of Africa, America, Borneo, India and Sri Lanka (Chung *et al.*, 2008; Tiwari *et al.*, 2011). *Spilanthes acmella* var *oleracea*, also known as *Acmella oleracea*, *Spilanthes acmella* and most commonly as jambú, toothache plant, Brazil cress, and Paracress, is a plant original from the tropics of Asia and South America (Chung *et al.*, 2008; Prachayasittikul *et al.*, 2013). Previous studies

have assessed *S. acmella* extract bioactivity as analgesic and local anaesthetic in rats and mice (Ansari *et al.*, 1988; Nomura *et al.*, 2013) and leaves of the plant were investigated for anti-inflammatory properties (Barman *et al.*, 2009). Jambu extracts also proved to possess anti-hyperalgesic activity (Ratnasooriya and Pieris, 2005). The presence of N-alkylamides in jambu, namely "spilanthol" (N-isobutyl-2E,6Z,8E-decatrienamide) suggests that the therapeutic effect observed is a result of its marked anaesthetic activity (Nomura *et al.*, 2013).

The transport of live fish has increased along the growth of aquaculture production (Stieglitz *et al.*, 2012). The deterioration of water quality aggravates the stress condition in fish during transport and in various cases is a limiting factor (Emata, 2000; Ross and Ross, 2008). Since transport acts as a potential stressor that adversely affects the health and welfare of fish, anaesthetics are frequently added to the water to minimize stress and mortalities during and post-transport (Iversen *et al.*, 2009; King, 2009).

Juvenile fish are usually transported in plastics bags inflated with pure oxygen and consequently there is a rapid increase in dissolved oxygen concentrations in the transport water (Golombieski *et al.*, 2003). It is common knowledge that increases in environmental oxygen concentrations can lead to increased reactive oxygen species (ROS) generation (Halliwel and Gutteridge, 1999; Hermes-Lima, 2004; Lushchak, 2011).

Tambaqui *C. macropomum* is widely farmed in several countries of South and Central America (Sevilla and Gunther, 2000; Valenti *et al.*, 2000). This species shows good tolerance to oxygen changes in water, and in natural environment this parameter changes markedly during the daytime and seasonal water level oscillations in the Amazon Basin. High antioxidant levels in liver and blood of wild tambaqui have been described (Marcon and Wilhelm Filho, 1999), which were regarded as effective in preventing or attenuating oxidative stress in natural environment. Those high basal levels are possibly a result from the marked changes in oxygen availability, temperature and pH that are frequently observed in Amazon Basin waters, especially during short-term periods of transition from hypoxia to hyperoxia (Marcon and Wilhelm Filho, 1999).

To date, there are no reports about anaesthetic/sedative activity of EO of *N. grandiflora* in fish and no information is available about the use of waxy extract of *S. acmella* in the transport of this species. Moreover, antioxidant competence of tambaqui submitted to transport in hyperoxic conditions is unknown.

The aim of this study was to evaluate the anaesthetic activity of EO of *N. grandiflora* and waxy extract of *S. acmella* in juvenile tambaqui as well as to investigate the effect of both plant extractives on oxidative stress status of fish submitted to transport.

2. Materials and Methods

2.1. Animals

Tambaqui juveniles were purchased from a commercial fish farm in the State of Amazonas, Northern Brazil and transported to the laboratory where they were maintained in continuously aerated 250 L tanks with controlled water parameters. All fish were acclimated for 15 days in two similar semi-static systems (for experiments 1 and 2) and photoperiod was fixed at 12 L/12 D. Fish were fed twice a day at 2% of biomass with commercial feed (28% crude protein). Thirty minutes after the feeding, the faeces and food remains were siphoned out. The daily volume of water withdrawn during the siphoning was about 20 to 40% of the total volume of the tanks, and the same volume was immediately replenished at the same conditions of the water that was in the tanks before feeding. Juveniles were fasted for a period of 24 h prior to the experiments. The methodologies of the experiments were approved by the Ethical and Animal Welfare Committee of the Universidade Federal do Rio Grande - FURG.

2.2. Plant materials

Essential oils or waxes such as those obtained from *N. grandiflora* and *S. acmella*, respectively, are poorly diluted in water and therefore pre-dilution in ethanolic vehicle was performed before using in the trials.

The EO of *N. grandiflora* was extracted by hydrodistillation using a Clevenger type apparatus for 3 h (Sartor, 2009). A pool from three EO samples, obtained from the leaves of three specimens was prepared. On the day of the trials, the EO pool was pre-diluted in ethanol (96%) at a ratio of 1:9, (1 mL of EO diluted in 9 mL of ethanol) so

that each mL of the solution contained 100 μL of EO. The stock solution was stored at 4 ºC in amber glass bottles until biological tests. For more details on the extraction methodology and the EO components see Silva (2013).

Samples of the waxy extracts of the flowers of *Spilanthes acmella* var *oleracea* (L.) were obtained by fractionated supercritical fluid extraction methodology with the use of CO_2 [SFE(CO_2)]. For details on the methodology of extraction and the chemical composition of the extract see Dias *et al.* (2012). The stock solution was prepared by weighing and diluting the jambu flowers extract in ethanol (96%) yielding a 2.24 mg mL^{-1} solution which was stored in an amber glass bottle at 4°C until its use.

2.3. Biological activity

Experiment 1: Anaesthetic efficacy of the EO of *N. grandiflora* and anaesthesia with extract of Jambu, *S. acmella* .

Juvenile fish (3.3 ± 0.5 g; 5.5 ± 0.8 cm, total length) irrespective of sex were transferred to aquaria containing 30 L of continuously aerated water. The same water from the recirculation system that specimens were acclimated was used for anaesthesia tests. Concentrations of EO of *N. grandiflora* at 25; 50; 100; 200; and 300 μL L^{-1} and [SFE(CO_2)] compound of *S. acmella* at 5; 10; 15; 20; and 25 mg L^{-1} were used in this experiment according to preliminary tests carried out to determine the appropriate concentration ranges. An ethanol control (EC) was also established at the same concentration of the highest anaesthetic concentration used. To evaluate the time required for anaesthesia induction 10 juveniles were used for each concentration tested and each juvenile was a replicate and used only once. All animals were starved for 24h prior to the tests. Times (in sec.) to reach the different stages of induction and recovery were cumulative and recorded with a digital stopwatch. The characterization of the stages was considered as described by Park *et al.* (2008) with modifications and is shown in Table 1.

The maximum observation time was 30 min. After induction, juveniles were transferred to anaesthetic-free tanks and the time elapsed for recovery was registered. Animals were considered recovered when they showed normal swimming behaviour in response to external stimuli. After recovery, fish were grouped according to the

anaesthetic protocol and transferred into continuously aerated 250 L tanks, where they were observed for at least 48 hours to check for mortalities.

Experiment 2: Transport and evaluation of oxidative stress indicators.

Tambaqui juveniles (8.6 ± 0.6 g; 8.1 ± 0.4 cm, total length), irrespective of sex, were used for this experiment. According to experiment 1, two groups of fish (n=10/group) were exposed for 10 min to concentrations corresponding to 10 up to 15% of the pre-established anaesthetic concentrations of *S. acmella* extract and *N. grandiflora* EO, respectively, which induced only slight anaesthesia in this species, clinically characterized by lethargy and reduced response to external stimuli. Those sedation concentrations were used in the transport water and corresponded to 1 mg L^{-1} and 30 µL L^{-1} of *S. acmella* extract and *N. grandiflora* EO, respectively.

Juveniles were weighed and placed in plastic bags (5 L) with 1.5 L of water that were inflated with pure oxygen and tied with rubber strings. The load density was set at approximately 160 g L^{-1} (27 specimens per bag and bags were in triplicate per group), which corresponds to the density used in commercialization of this species in the Amazon region. Fish were transported on paved road for 2, 6 and 10 h inside these bags in the presence or absence of the [SFE(CO_2)] extract of *S. acmella* or EO of *N. grandiflora* in water (1 mg L^{-1} and 30 µL L^{-1}, respectively).

At the end of each period (2; 6; and 10 h), 30 specimens per group (10 per bag) were taken and killed by spinal sectioning. Muscle, gills, liver and brain were removed and tissue pools were prepared out of every five specimens, yielding six tissue pools per group (2 per bag); each tissue pool was weighed and immediately stocked in a freezer at − 80 °C for subsequent analysis of total antioxidant capacity (ACAP), GST activity and lipoperoxidation (LPO) damage measured by thiobarbituric acid reactive substances (TBARS) in organs. Pools of tissues from non-transported fish (30 specimens) from the acclimation tanks in normoxic conditions and not exposed to the anaesthetics were used to provide initial reference values of antioxidant status and LPO levels.

Water parameters: temperature, alkalinity, dissolved oxygen, pH and total ammonia were determined at the beginning (0 h) and at the end of each transport (2; 6; and 10 h).

2.4. Water Quality

For experiments 1 and 2 water parameters (mean ± SD) during acclimation were maintained as follows: 7.40 ± 0.32 mg L^{-1} and 7.90 ± 0.50 mg L^{-1} of dissolved oxygen (DO) respectively; 26.5 ± 0.4 °C and 26.8 ± 0.6 °C temperature respectively, measured with an oxygen meter (Yellow Springs Instruments, USA). Values of pH were 6.8 ± 0.1 and 7.1 ± 0.3 respectively, measured with a pH meter (Five Easy FE20, Switzerland). Total ammonia nitrogen (TAN) values were 0.9 ± 0.1 mg L^{-1} $NH_4^+ + NH_3^-$ - N and 0.6 ± 0.1 mg L^{-1} $NH_4^+ + NH_3^-$ - N respectively, and were quantified according to Unesco (1983); nitrite 0.03 ± 0.01 mg L^{-1} and 0.06 ± 0.01 mg L^{-1} respectively and were determined according to Bendschneider and Robinson (1952). Total alkalinity of 60.5 ± 0.2 and 62.7 ± 0.5 mg $CaCO_3$ L^{-1} respectively, and were evaluated by titration in accordance with Eaton *et al*. (2005) guidelines.

2.5. Oxidative stress parameters

Briefly, tissues were homogenized at a rate of 1:5 (w v^{-1}) in buffer (Tris-HCl – 100 mM; EDTA – 2 mM; and $MgCl_2.6H_2O$ – 5mM) (Da Rocha *et al.*, 2009). The supernatants resulting from the centrifugation of the homogenates (10,000 x*g*, 20 minutes (4ºC) were used for all analyses. After homogenization, total protein content was determined through Biuret method. Thereafter, samples of muscle, gill and liver were diluted with buffer to 2.0 mg protein mL^{-1} and brain to 0.5 mg protein mL^{-1} for posterior analysis of total antioxidant capacity against peroxyl radicals.

Total antioxidant capacity against peroxyl radicals was determined according to the method described by Amado *et al.*, (2009). This method consists of finding the antioxidant capacity of tissues using a fluorescent substrate (2′,7′ dichlorofluoresceindiacetate - H_2DCF-DA) and the production of peroxyl radicals by thermal decomposition of ABAP (2,2′-azobis 2 methylpropionamidinedihydrochloride). The fluorescence was determined through a microplate reader (Victor 2, Perkin Elmer), at 37ºC (excitation: 485 nm; emission: 530 nm) with readings at every 5 minutes, during 30 minutes. The results were expressed as a relative area (the difference between the area with and without ABAP divided by the area without ABAP). Using this methodoly a low relative area means high total antioxidant capacity.

The lipoperoxidation of all tissues was measured using the methodology described by Oakes and Van Der Kraak, (2003). To determine TBARS by the quantification of MDA (malondialdehyde), 20 µl of BHT solution (67µM), 150 µl 20% acetic acid solution, 150 µl 0.8 % TBA solution, 50 µl Milli-Q H_2O, and 20 µl of 8.1% SDS were added to 30 µl of each sample homogenate before being heated at 95 ºC with water bath for 30 minutes. Thereafter, 100 µl of Milli-Q H_2O and 500 µl of n-butanolwere added to the final solution. The remaining supernatant of centrifugation (3000 rpm, 10 minutes, 15 ºC) was used to determine the fluorescence (excitation: 520 nm; emission: 580 nm) and the results were expressed as nmol TMP mg protein^{-1}, where TMP stands for the standard Tetramethoxypropane (ACROS Organics).

Determination of glutathione-S-transferase (GST) activity was performed according to Habig *et al.* (1974). Potassium phosphate buffer (KH_2PO_4 – 0.05 M; K_2HPO_4 – 0.05 M; and Milli-Q H_2O; pH = 7.0) was used as reaction medium (previously heated at 25 ºC with water bath) and then it was added substrate 1-chloro-2,4-dinitrobenzene (CDNB) (50 mM) and reduced glutathione (GSH, 25 mM). CDNB and GSH were added together with 10 µl of sample homogenate in transparent 96 wells microplate and read at 340 nm using microplate reader (Victor 2, Perkin Elmer). The results were expressed as nmol of conjugated CNDB-GSH mg protein^{-1} min^{-1}.

2.6. Data analysis

All data are presented as mean ± SD. To verify the normality and homogeneity of variances, data were submitted to Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA and Tukey tests were used for data of anaesthesia induction and recovery times (experiment 1). Two-way ANOVA (with transport length and anaesthetic being used as factors) and Tukey tests were performed to check differences between water quality variables and oxidative stress parameters in experiment 2. Water quality parameters before and after transport were submitted to repeated measures ANOVA and Dunnet test. The non-parametric Kruskal–Wallis test was used (Zar, 1996) to test the results obtained for ACAP and GST activity in liver, which did not show homogeneity of variances. Trends of the anaesthesia stages and recovery were fitted using both linear and non-linear response models with measured

times to reach stages being the response variables and concentration of the anaesthetics the predictor variables. Minimum significance level was set at $p<0.05$ in all cases.

3. Results

No mortalities were observed until two weeks after the trials among specimens used in experiment 1 and among animals that were not used for tissue samplings and returned to the tanks of origin in experiment 2.

3.1. Anaesthetic efficacy

Baths with EO of *N. grandiflora* did not promote agitation (A1) behaviour at any concentration tested. The higher the concentration the longer it took for stage A2 (see Table 1 for definition of each stage) to be observed, being the times at the dosages of 200 and 300 μL L^{-1} (186 and 880s, respectively) significantly different ($p<0.05$) between each other and both concentrations took significantly longer times for the specimens to reach stage A2 when compared to dosages of 25 up to 100 μL L^{-1} (Table 2). Only concentrations at 200 and 300 μL L^{-1} were effective in inducing anaesthesia (stage A3) with loss of reaction to tail pinch stimulus and irregular or minimum opercular beating. Fish exposed to the concentration of 100 μL L^{-1} or below did not reach full anaesthesia within 30 min (1,800 s) of observation (Table 2).

Fish anaesthetized with extract of *S. acmella* showed a shorter time ($p<0.05$) to reach the stage of agitation (A1) at the concentration of 10 mg L^{-1} and forward compared to the lower concentration used. The same pattern was noticed for stage A2 and the higher the concentration the faster the behavioural characteristics of this stage were observed, being the time at the dosage of 10 mg L^{-1} or above significantly shorter ($p<0.05$) to show loss of equilibrium compared to the lowest dosage tested. All the tested concentrations were effective in inducing anaesthesia (stage A3) with loss of reaction to tail pinch stimulus and irregular or minimum opercular beating. Fish exposed to the lowest concentration (5 mg L^{-1}) reached anaesthesia in 6.8 min (408 s) and the subsequent increasing concentrations tested reduced the induction time to a minimum of 44% thereof.

Based on visual assessment fish were acknowledged fully recovered after re-establishment of equilibrium and normal swimming for all concentrations tested

regardless of anaesthetic used. At concentrations of 25 and 50 μL L^{-1} of EO of *N. grandiflora* recovery was significantly faster compared to recovery of animals exposed to higher concentrations. Fish exposed to 10 mg L^{-1} of *S. acmella* extract recovered faster from anaesthesia compared to all other concentrations used (Table 2).

The overall trends observed as a result of the relation between the time required for reaching the different stages of induction or recovery from anaesthesia over the concentrations tested are shown in equations in Table 2 for both anaesthetic compounds ($p<0.05$).

Water comprised solely of ethanol addition at the highest concentration used to dilute the essential oil of *N. grandiflora* (2700 μL L^{-1}) did not produce any anaesthetic effect on tambaqui juveniles after 30 min observation.

3.2. Water quality

Overall means for water quality before and after transport are presented in Table 3. After 6 and 10 h of transport, irrespective of group, all parameters of water quality were significantly different from their respective reference values before transport, except for the alkalinity which showed differences only after 10 h of transport with EO of *N. grandiflora* and no changes in the water added with extract of *S. acmella* were observed at any given transport time. Most of the variables of water quality after 2 h of transport were not significantly different from their respective reference values before transport. The exceptions were total ammonia and the persistence of hyperoxic conditions in the water after 2 h transport in the groups without compound and with *N. grandiflora* EO.

No differences were observed in the water quality parameters between groups within the same time of transport, except for the decreased DO levels in the water of bags transported for 2 h with *S. acmella* extract.

Water pH in the bags transported with the anaesthetics decreased ($p<0.05$) after 6 and 10 h when compared to water pH of bags transported for 2 h. As for the bags transported without anaesthetics only after 10 h of transport the water pH significantly decreased ($p<0.05$) when compared to 2 h of transport. Temperature decreased ($p<0.05$) after 10 h of transport with *N. grandiflora* EO when compared to 2 h of transport and decreased after transport for 10 h in the bags without anaesthetics when compared to

transports for 2 and 6 h also without anaesthetics; no differences ($p>0.05$) were observed in the temperature of the water with *S. acmella* extract within this group. Furthermore, no differences ($p>0.05$) were observed for alkalinity or dissolved oxygen levels between transport times, except for the DO after 2 h transport with *S. acmella* extract.

Total ammonia was increased ($p<0.05$) for all groups, regardless of the presence of the anaesthetics compared to the water of non-transported fish. However, no increases ($p>0.05$) in this parameter were observed over transport time.

3.3. Oxidative stress status in organs

3.3.1. Muscle

Total antioxidant capacity was lower in controls transported for 6 and 10 h compared to controls after 2 h of transport (Fig 1A). ACAP was higher (lower relative area) after 2 h of transport in controls compared to animals transported in the presence of both anaesthetics within the same transport time, whereas the opposite occurred within 10 h: higher antioxidant capacity ($p<0.05$) was observed in muscle from fish exposed to both anaesthetics when compared to their respective control. No differences ($p>0.05$) were observed in ACAP between groups transported for 6 h (Fig 1A).

Animals transported for 2 h in the presence of both anaesthetics showed similar activity of GST compared to transported controls ($p>0.05$, Fig 1B). After 6 h of transport in the presence of *S. acmella* extract, muscle showed less activity of GST compared to control group within the same transport time ($p<0.05$). Increased enzyme activity was observed after transport for 10 h in the presence of *N. grandiflora* EO compared to control group within the same transport time ($p<0.05$). All groups showed significant decreased activity of GST ($p<0.05$) compared to non-transported controls, except for the group transported with *N. grandiflora* extract for 10 h (Fig 1B).

No changes in TBARS levels were observed in transported controls between transport times (Fig 1C). Peroxidation of lipids was lower after 6 h for fish transported with both anaesthetics compared to their respective control within transport time and also lower compared to transports after 2 h with *S. acmella* or after 10 h with *N. grandiflora* extracts. TBARS levels were decreased after transport for 6 h with both

anaesthetics and after 2 and 10 h with EO of *N. grandiflora* and extract of *S. acmella* respectively, compared to non-transported animals in normoxia ($p<0.05$, Fig 1C).

3.3.2. Gills

ACAP in gills was not significantly different ($p>0,05$) over time in controls, but it was higher in fish transported for 6 and 10 h in the presence of both anaesthetics compared to ACAP in fish transported for 2 h in the presence of both compounds ($p<0.05$, Fig 2A). Only after 2 h transport with EO of *N. grandiflora* ACAP was significantly higher ($p<0.05$) compared to gills of non-transported fish. No differences ($p>0.05$) were observed between groups within the same transport time (Fig 2A).

GST activity was not significantly different ($p>0.05$) within each transport time or over time for all groups. Nevertheless, the enzymatic activity increased ($p<0.05$) in controls after 6 and 10 h transport compared to non-transported group (Fig 2B).

TBARS levels were higher in controls after 10 h transport compared to those transported for 2 h ($p<0.05$, Fig 2C) and it was significantly lower after 10 h transport in the presence of *N. grandiflora* EO compared to controls within the same transport time. Animals transported for 10 h with EO of *N. grandiflora* showed lower TBARS levels compared to animals transported for 6 h with the same EO. Fish transported for 6 h with both anaesthetics and animals transported for 10 h in control group or in the presence of *S. acmella* showed higher TBARS levels compared to non-transported fish ($p<0.05$, Fig 2C).

3.3.3. Liver

Total antioxidant capacity was higher in controls transported for 10 h compared to those transported for 2 or 6 h ($p<0.05$, Fig 3A). The opposite pattern was observed for transport in the presence of *S. acmella* extract in which ACAP was higher after 2 h transport and lower after transports of 6 or 10 h ($p<0.05$). ACAP was higher after 2 h of transport with *S. acmella* extract compared to control group within the same transport time and increased ($p<0.05$) after 6 h in group transported without anaesthetics. ACAP decreased after 6 h transport in the presence of extract of jambu and also in controls after 2 and 6 h of transport compared to non-transported animals ($p<0.05$). No differences were observed in ACAP among groups transported for 6 h ($p>0.05$, Fig 3A).

Fish transported with both compounds showed higher GST activity after 10 h compared to controls within the same time or non-transported fish ($p<0.05$) (Fig 3B). Lipoperoxidation levels in liver were not significantly different ($p>0.05$) either between transport times or within each transport time for all groups, except for transport with *S. acmella* extract between 6 and 10 h in which TBARS concentration was lower ($p<0.05$) in the latter. No changes ($p>0.05$) were observed in TBARS for all groups compared to non-transported controls (Fig 3C).

3.3.4. Brain

ACAP was lower (higher relative area) in controls transported for 2 h compared to controls transported for 6 h and also compared to those transported in the presence of both anaesthetics within the same transport time. ACAP increased in the brain of fish transported for 10 h in the presence of *S. acmella* extract compared to non-transported control ($p<0.05$, Fig 4A). No significant alterations ($p>0.05$) were observed in GST activity (Fig 4B) or lipoperoxidation levels (Fig 4C) in the brain of fish for all groups either between transport times or within each transport time.

4. Discussion

Concerns have been raised that some anaesthetics may cause an aversive reaction and irritation of tissues in fish following exposure to the drug (Reed and Jennings, 2011; Williams *et al.*, 2009). Adverse reactions have been reported during initial stages of anaesthesia in fish (APC, 2009). Aversive reactions are characterized as "coordinated excitatory behaviour with increased respiratory rate" followed by "violent thrashing and jumping" before eventually loosing equilibrium (Lewbart, 1998). Those aforementioned adverse reactions, hereby characterized as Agitation (A1) were not present in fish exposed to EO of *N. grandiflora*.

Time to loss of equilibrium (A2) was unusually increased in specimens exposed to higher concentrations of this compound. Contradictorily, lower concentrations from 25 to 100 μL L^{-1}, incapable of inducing deep anaesthesia, caused fish to lose equilibrium (A2) faster than subsequent higher concentrations of 200 and 300 μL L^{-1}. Anaesthesia (A3) was achieved only for the two highest concentrations, however the oil did not promote fast anaesthesia within the recommended maximum induction time of 3

minutes (180 s) (Bell, 1987; Marking and Meyer, 1985). Sladky *et al.*, (2001) verified that fish that had rapid inductions at high concentrations of eugenol were more likely to recover uneventfully.

Fish were fully recovered after being exposed to all concentrations of EO of *N. grandiflora*. However, only at the anaesthetic concentration of 200 μL L^{-1} recovery occurred within the maximum recommended time of 5 min (300 s). No recovery was observed within 5 min for the anaesthetic concentration of 300 μL L^{-1}, therefore not complying with the recommended maximum time for recovery from deep anaesthesia in this case (Bell, 1987; Iwama and Ackerman, 1994; Marking and Meyer, 1985; Ross and Ross, 2008).

During agitation fish were hyperventilating, "coughing", surfacing and frantic swimming was observed. Hyperactivity is usually a response of only a few seconds to the slightly irritant properties of the drug and has been previously reported in literature (Ross and Ross, 2008). This state suggests transitory distress in juvenile tambaqui in this study. Similar behaviour was observed for the same species anaesthetized with benzocaine (Gomes *et al.*, 2001). Extract of jambu is a natural compound and its probability of causing more pronounced or less severe aversion in fish when compared to other synthetic or natural anaesthetics has not been investigated yet.

All five concentrations tested promoted anaesthesia with minimum opercular beating and loss of reaction to tail pinch stimulus (A3) within the stipulated observation time endpoint (30 min). Animals reached fast anaesthesia (A3) in around 3.0 min (182 s) at the concentration of 10 mg L^{-1}, complying with the recommended maximum induction time of 3 min (180 s) and recovery within 5 min (300 s) (Bell, 1987; Iwama and Ackerman, 1994; Marking and Meyer, 1985; Ross and Ross, 2008).

Although concentrations above 10 mg L^{-1} of *S. acmella* extract were also effective to induce fast anaesthesia within 3 min, this is the most suitable anaesthetic concentration to be recommended since from an economic perspective anaesthetics may be expensive and unnecessary higher concentrations will increase operating costs (Roubach *et al.*, 2001).

The recovery times were within the recommended limits for all concentrations except for the highest concentrations of extract of *S. acmella*. However, no linear pattern was observed for recovery times in anaesthesia with this extract as a function of

the concentrations tested and such a relatively irregular response for recovery may occur in fish submitted to anaesthesia baths (Ross and Ross, 2008).

A variety of other anaesthetics have been previously tested for tambaqui with recommended concentrations presented in Table 4. Appropriate anaesthetic activity of these different compounds was obtained with concentrations far higher than those of *S. acmella* extract used in the present study. Nonetheless, care should be used with comparisons between anaesthetic concentrations of the different anaesthetic agents, since the pharmacological presentation and exposure route can influence anaesthesia outcome (Ross and Ross, 2008) and different methodologies of extraction of natural compounds even from plants of the same species can yield significantly different amounts of bioactive products per gram of extract (Dias *et al.* 2012).

The anaesthetic activity of jambu extract verified in this study corroborates the findings of Barbas *et al.* (*non-published data*) as to the efficacy of *S. acmella* extract which was capable of inducing fast anaesthesia within 3 min at the concentration of 20 mg L^{-1} in juvenile tambaqui. However, only half (10 mg L^{-1}) of that anaesthetic concentration was necessary in this study to promote fast anaesthesia in less than 3 min and allow recovery within the recommended time of 5 min. Experimental conditions were similar, except for the weight/size of the specimens. This could indicate an influence of the body weight (Ross and Ross, 2008; Sneddon, 2012) since the animals used in this study were approximately 14-fold smaller in weight and therefore could be more sensitive to anaesthesia induction. Small fish may absorb more anaesthetic through the skin than large fish and therefore the total uptake is greater (Myszkowski *et al.*, 2003). Yet, larger fish have a smaller gill surface area in relation to body weight, and consequently a smaller area for anaesthetic diffusion (Zahl *et al.*, 2009).

Sedation or superficial anaesthesia was observed when fish were submitted to 1 mg L^{-1} of jambu extract and 30 µL L^{-1} of EO of *N. grandiflora*. These conditions are considered appropriate for non-invasive procedures such as biometrics, gill scrapes, tagging, or transport when only sedation is necessary (Sneddon, 2012).

Antioxidant enzyme activities and oxidative stress biomarkers are closely related and lipoperoxidation (LPO) products are involved in regulation or/and modification of some enzyme activities (Lushchak *et al.*, 2005; Lushchak and Bagnyukova, 2006a, 2006b). Antioxidant enzymes usually act in concert in order to ensure protection against

oxidative insult (Hermes-Lima, 2004; Storey, 1996). In turn, development of oxidative stress is evidenced by alterations in levels of oxidative damage markers. Products of protein and lipoperoxidation are the most commonly used being the latter frequently a quick response to ROS generation (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004; Stadtman and Levine, 2000; Storey, 1996).

Juvenile tambaqui transported in hyperoxic conditions with or without anaesthetics in this study showed different response patterns in antioxidant defense and different susceptibility to oxidative damage in organs as a function of transport duration as well.

Total antioxidant capacity in muscle was improved to at least 14.5% in the presence of the anaesthetics within 10 h transport time compared to their respective control, which could be an indication of enhanced oxidative defense in the muscle of fish exposed to these anaesthetics in transports of such a length of time. Antioxidant properties have been previously reported for both extractives tested (Dias *et al.*, 2012; Ribeiro *et al.*, 2005); Although GST activity showed considerable variation in muscle within each transport time and depending on the anaesthetic used, a higher ACAP in the muscle after 10 h transport with *N. grandiflora* EO was consistent with a higher (78%) GST activity at the same transport time for the same group. The antioxidant defense can be enhanced in fish muscle in the presence of this EO and it may have been an additional support in keeping TBARS concentration within reference values (non-transported fish in normoxia).

Muscle from fish transported with extract of *S. acmella* for 10 h showed a reduction of 33% in TBARS compared to non-transported fish kept in normoxic water, which is an indication of the potent effect of this extract to attenuate lipid peroxidation even in relation to resting fish, *i.e*, fish not exposed to transport in hyperoxia, which is a pro-oxidant condition.

Although no advantages for improving ACAP in muscle were observed after 2 or 6 h of transport in the presence of either anaesthetics compared to controls, response in muscle showed that both anaesthetics enhanced protection against lipid peroxidation mainly in fish transported for 6 h, decreasing TBARS levels to a minimum of 41.5% compared to fish transported without anaesthetics.

Similar results were observed in the muscle of silver catfish *Rhamdia quelen* transported for 6 h with 30 to 40 μL L^{-1} of *Aloysia triphylla* EO (Zeppenfeld *et al.*, 2014), where TBARS concentrations were decreased compared to controls transported without anaesthetic and thus, a potential protective effect in muscle could be observed as well.

Although ACAP in gills was lower in transport for 2 h with both anaesthetics compared to the transport for 6 h and 10 h in the presence of both anaesthetics, no differences were observed between controls at any given transport time and GST activity was not significantly affected in gills by the presence of the anaesthetics during transport whatsoever. Nevertheless, lipid peroxidation was significantly more severe (65%) in controls transported for 10 h compared to controls transported for 2 h and a protective effect was observed with the use of EO of *N. grandiflora* in the water of animals transported for 10 h since TBARS levels were significantly lower (39.5%) compared to controls. Azambuja *et al.* (2011) reported no differences in TBARS levels in gills of silver catfish transported for 5, 6 and 7 h without anaesthetic, however, similar to our findings, a protective effect of *Lippia alba* extract in gills of silver catfish transported for 7 h was observed with decreased TBARS concentration compared to fish transported without anaesthetic for the same length of time.

Variations in ACAP were observed in the liver within transport times in which total antioxidant capacity was either higher (58.5%) in fish transported for 2 h with *S. acmella* extract compared to controls or unexpectedly lower (at least 155%) in fish transported for 10 h in the presence of both anaesthetics compared to controls transported for the same time. Furthermore, opposite response patterns were established over time in which transported controls showed higher ACAP after 10 h transport compared to controls transported for shorter times whereas ACAP decreased in the liver of animals transported with both anaesthetics for 6 or 10 h compared to the same anaesthetic treatments in fish transported for 2 h. Although not significantly different over time, transport with *N. grandiflora* EO showed a similar pattern for ACAP compared to transports with *S. acmella* extract. No studies have been conducted to assess ACAP in liver of fish under similar circumstances, which makes it difficult to discuss these results. It is also unknown if GST is capable of conjugating molecules within the extracts which in turn could consequently affect ACAP results.

The addition of anaesthetics in the water did not affect liver ACAP in groups transported for 6 h and GST activity was only increased (58%) in fish transported for 10 h in the presence of the anaesthetics. ACAP in brain showed similar responses observed in muscle, however, it occurred in different transport times, nevertheless antioxidant capacity was enhanced in the presence of the anaesthetics.

No significant differences were observed within transport times in GST activity for most of the organs, except for muscle and liver, where a higher GST activity was observed in animals transported for 10 h in the presence of anaesthetics compared to their respective control. Differently, silver catfish transported for 5h in hyperoxic conditions with EO of *L. alba* (10 μL L^{-1}) exhibited significant decrease in hepatic and brain GST activity compared to the liver and brain of fish transported under normoxic conditions for 6 h in the presence of the anaesthetic and also compared to fish transported for the same length of time without anaesthetic (Azambuja *et al.*, 2011). Our findings are also different from the results observed in the liver of silver catfish, transported for 6 h in the presence of *L. alba* EO (30 μL L^{-1}), where GST activity was significantly decreased compared to fish transported without anaesthetic for the same time (Salbego *et al.*, 2014).

TBARS levels in brain and liver did not change irrespective of transport length or the presence of the anaesthetics in the water. Similarly, Azambuja *et al.* (2011) reported that lipid peroxidation did not change in brain or liver of silver catfish transported for 5 h in hyperoxic conditions without anaesthetics compared to fish transported in normoxic anaesthetic-free water for 6 h, however, TBARS levels were decreased in liver after transport in hyperoxia for 5 h in the presence of EO of *L. alba* compared to hyperoxic transport for the same length of time without anaesthetic.

Wilhelm Filho (2007) reported that inter-specific differences in antioxidant responses depend on quantitative distribution of antioxidant defenses in the different tissues. Intra-specific organ variations in ROS responses can be related to anatomical localization, exposure route and defense capacity (Ahmad *et al.*, 2006). Therefore, in the face of different pro-oxidant challenges, antioxidant status can vary from organ to organ as previously described in literature (Amado *et al.*, 2009; Da Rocha *et al.*, 2009; Monserrat *et al.*, 2008).

All transported groups were still exposed to hyperoxic concentrations at the end of the transport, except for the group transported for 2 h in the presence of *S. acmella* extract. One cannot rule out that such a reduction in oxygen concentration may also occur due to the oxidation of molecules within the extract. Nevertheless, oxygen concentration was still relatively high for this group at the end of the transport. All tissues, except for the muscle, showed either the same or increased GST activity compared to normoxia non-transported fish. The decreased GST activity in muscle for most of the groups compared to normoxic non-transported fish could be the result of an earlier and intense detoxification activity followed by a decrease in the synthesis of GST protein(s) at a molecular level (Gallagher and Sheehy, 2000) which in turn led to decreased activity of this enzyme by the time of the first sampling of muscle tissue.

Glutathione-S-transferasecatalyzes the conjugation of several xenobiotics with glutathione (GSH) as part of the detoxification process (Habig *et al.,* 1974) and also for the protection of cells against by-products originated from the peroxidation of tissues (Van der Oost *et al.*, 2003). Presumably, due to the high sensitivity of biotransformation enzymes (Van der Oost *et al.*, 2003), the overall variations in GST activity between normoxia non-transported fish and transported groups could be not only the consequence of differences in oxygen levels but also differences between experimental conditions of transported *vs.* non-transported animals, coupled with changes in other water quality parameters, *e.g.* temperature (Lushchak, 2011; Lushchak and Bagnyukova, 2006a, 2006b;) in the course of the transport or other unknown variables.

ACAP in muscle could not be compared to reference values of normoxia non-transported fish, due to the loss of those samples. As to the other tissues, no marked changes were observed in ACAP between groups transported in hyperoxia and normoxia non-transported fish regardless of transport time or the presence of the anaesthetic, except for the liver, where most of the groups showed decreased total antioxidant capacity when submitted to transports in this study, which is an indication that transport generates a pro-oxidant condition.

The exposure to hyperoxia induced oxidative stress in different fish species such as goldfish *Carassius auratus* (Lushchak *et al.*, 2005), atlantic salmon *Salmosalar* (Olsvik *et al.*, 2005) and senegal sole *Solea senegalensis* (Salas-Leiton *et al.*, 2009). Similarly, gills of tambaqui transported in hyperoxia for 6 and 10 h with or without

anaesthetics, showed higher levels of TBARS compared to those of non-transported fish under normoxia.

The results of all studied organs indicated that both anaesthetics improved the antioxidant defense in juvenile tambaqui submitted to transport, mainly in muscle and gills where a protective effect was observed after 6 and 10 h of transport in terms of LPO levels.

Anaesthesia with both compounds was achieved and transport of juvenile tambaqui in hyperoxia seemed to elicit transient antioxidant response changes and oxidative stress in tissues, which were ameliorated by the use of the anaesthetics. Although EO of *N. grandiflora* was capable of promoting deep anaesthesia, a fast induction response was not observed, however, this oil was effective for sedation and transport of juvenile tambaqui.

In conclusion, the extract of jambu flowers, *S. acmella* and EO of *N. grandiflora* proved to be suitable anaesthetics for juvenile tambaqui, promoting anaesthesia required for handling and other more invasive procedures. Furthermore, among the concentrations tested, the use of 10 mg L^{-1} extract of *S. acmella* was enough to induce fast anaesthesia with uneventful recovery. Sedation concentrations at 1 mg L^{-1} and 30 µL L^{-1} of *S. acmella* extract and *N. grandiflora* EO respectively, are recommended for transport of this species since those compounds enhanced protection against oxidative damage mainly in muscle and gills.

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Figure captions

Figure 1. Total antioxidant capacity (A)$^{+}$, GST activity (B) and lipid peroxidation levels (C) in muscle of juvenile tambaqui, *Colossoma macropomum* submitted to different transport times (2, 6 and 10 h) with and without anaesthetics. Values are expressed as means ± SD. When present, different letters indicate significant differences between transport time; * indicates significantly different from fish transported without anaesthetics (control) within the same transport time; means represented by open markers are significantly different from non-transported fish kept in normoxic conditions ($p<0.05$), n=30.

$^{+}$Samples of non-transported fish were lost

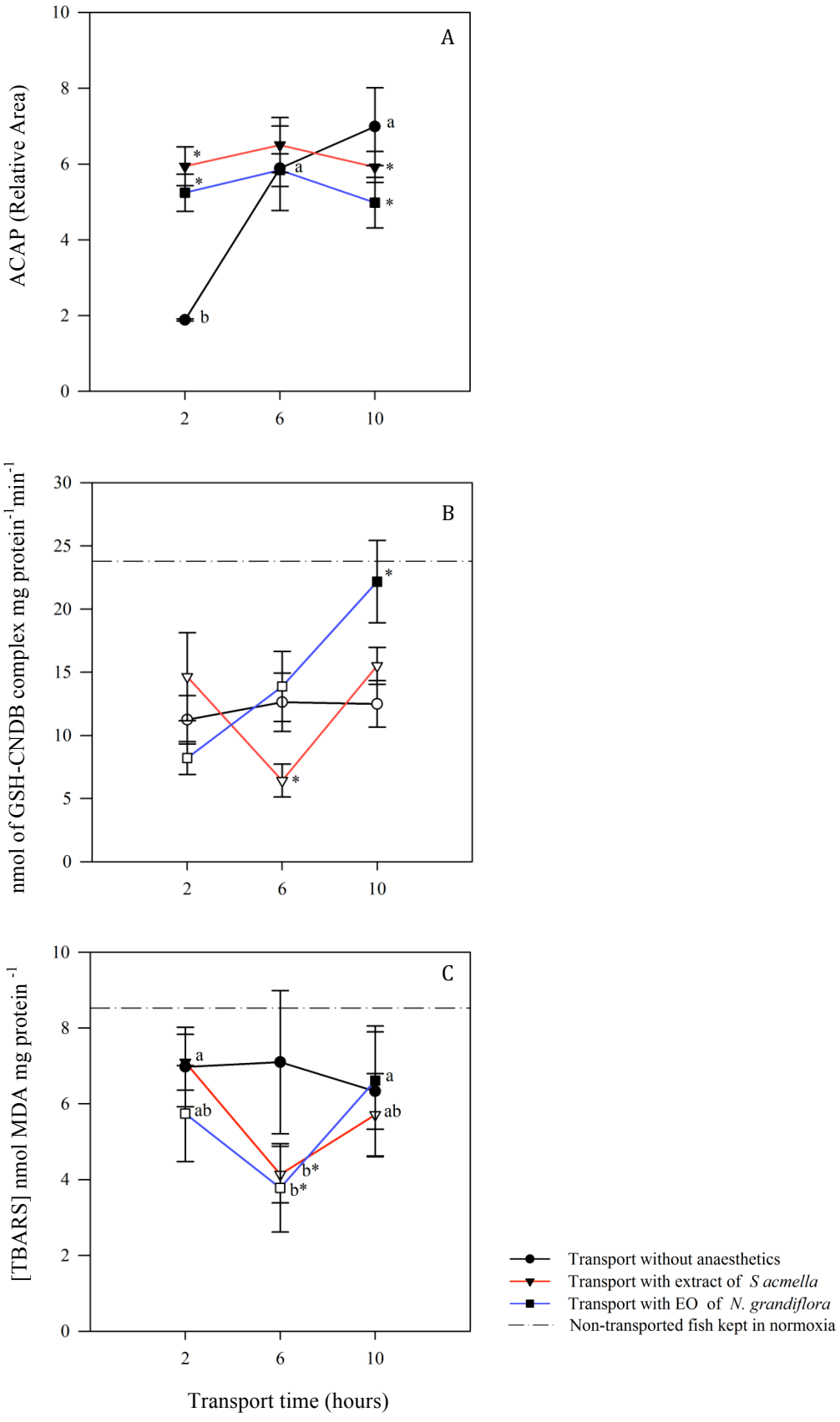
Figure 2. Total antioxidant capacity (A), GST activity (B) and lipid peroxidation levels (C) in gills of juvenile tambaqui, *Colossoma macropomum* submitted to different transport times (2, 6 and 10 h) with and without anaesthetics. Values are expressed as means ± SD. When present, different letters indicate significant differences between transport time; * indicates significantly different from fish transported without anaesthetics (control) within the same transport time; means represented by open markers are significantly different from non-transported fish kept in normoxic conditions ($p<0.05$), n=30.

Figure 3. Total antioxidant capacity (A), GST activity (B) and lipid peroxidation levels (C) in liver of juvenile tambaqui, *Colossoma macropomum* submitted to

different transport times (2, 6 and 10 h) with and without anaesthetics. Values are expressed as means ± SD. When present, different letters indicate significant differences between transport time; * indicates significantly different from fish transported without anaesthetics (control) within the same transport time; means represented by open markers are significantly different from non-transported fish kept in normoxic conditions ($p<0.05$), n=30.

Figure 4. Total antioxidant capacity (A), GST activity (B) and lipid peroxidation levels (C) in brain of juvenile tambaqui, *Colossomamacropomum* submitted to different transport times (2, 6 and 10 h) with and without anaesthetics. Values are expressed as means ± SD. When present, different letters indicate significant differences between transport time; * indicates significantly different from fish transported without anaesthetics (control) within the same transport time; means represented by open markers are significantly different from non-transported fish kept in normoxic conditions ($p<0.05$), n=30.

Figure 1


A
ACAP (Relative Area)
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8
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a
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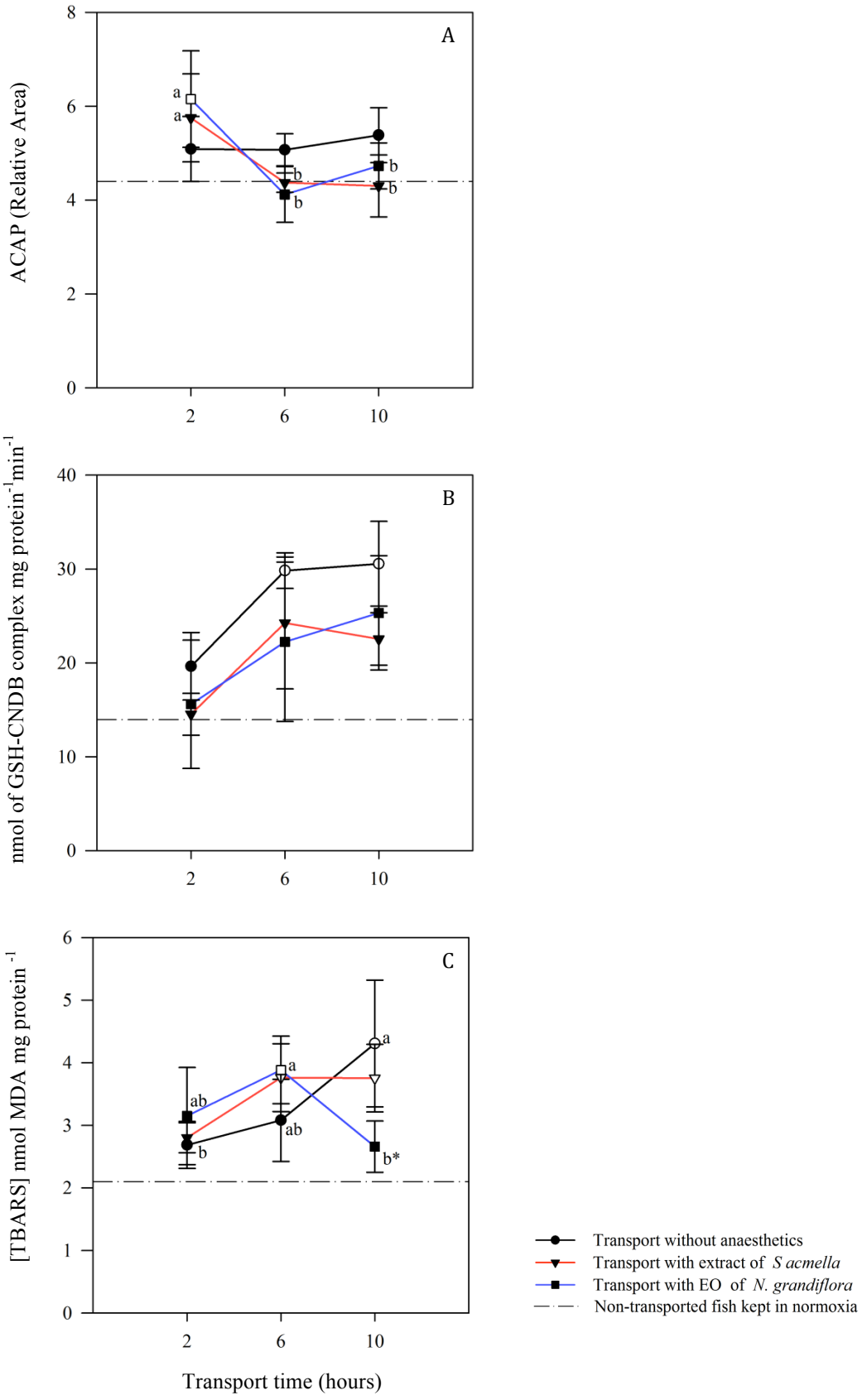
Transport time (hours)
Transport without anaesthetics
Transport with extract of S acmella
Transport with EO of N. grandiflora
Non-transported fish kept in normoxia

Figure 2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ACAP (Relative Area)
nmol of GSH-CNDB complex mg protein-1min-1
[TBARS] nmol MDA mg protein -1
Transport time (hours)
Transport without anaesthetics
Transport with extract of S acmella
Transport with EO of N. grandiflora
Non-transported fish kept in normoxia

Figure 3

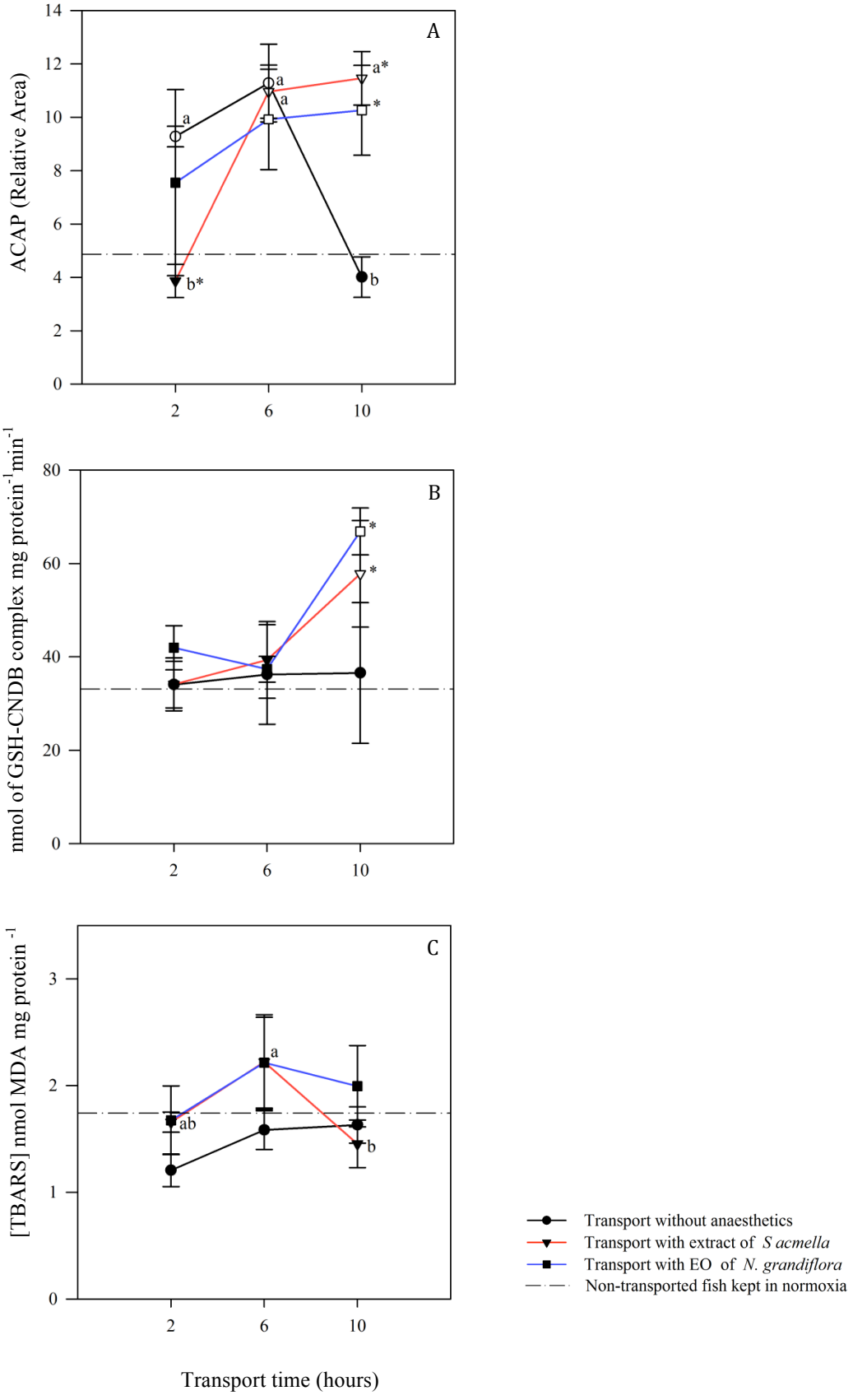
A
ACAP (Relative Area)
B
nmol of GSH-CNDB complex mg protein-1min-1
C
[TBARS] nmol MDA mg protein -1
Transport time (hours)
Transport without anaesthetics
Transport with extract of S acmella
Transport with EO of N. grandiflora
Non-transported fish kept in normoxia

Figure 4

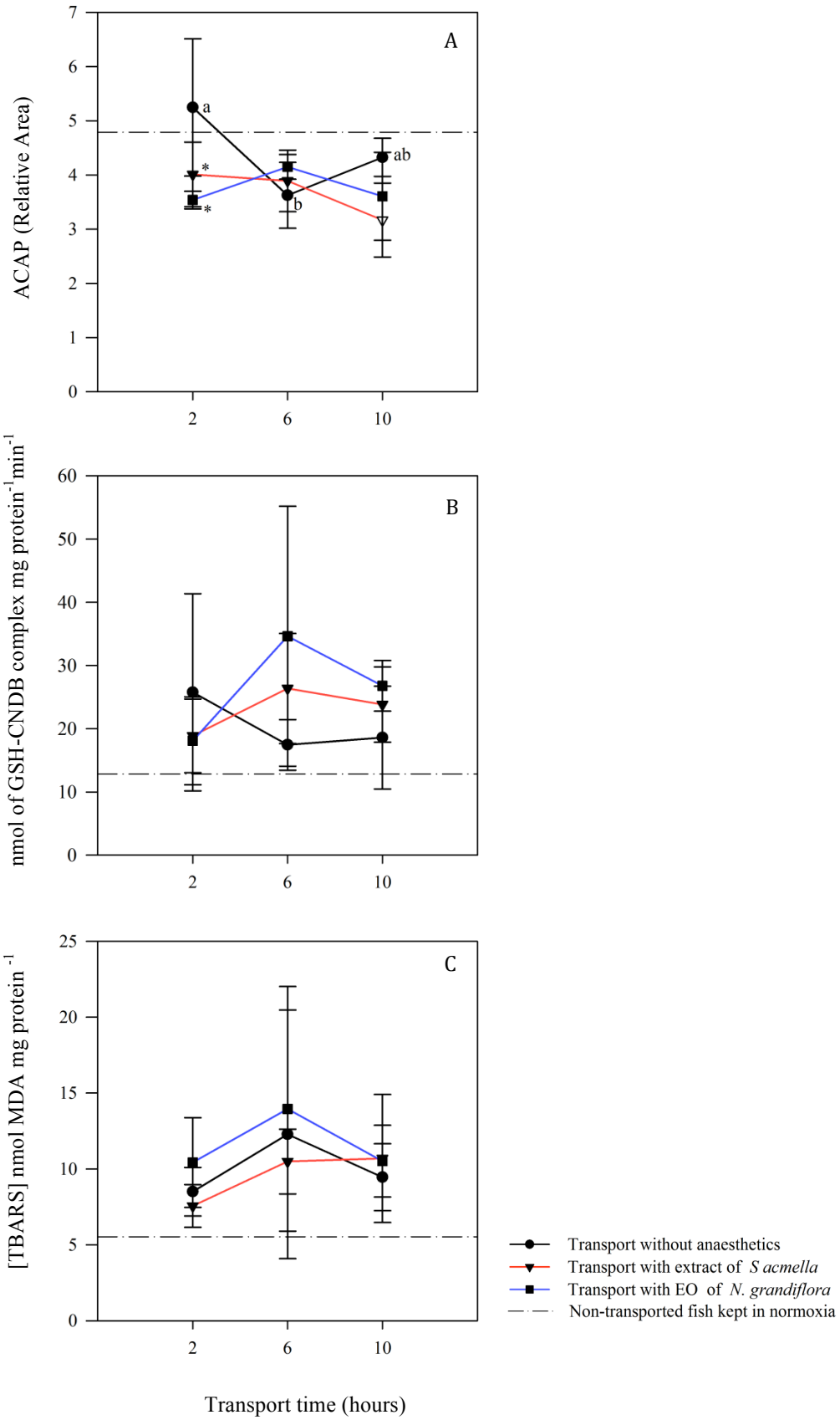
A
B
C
ACAP (Relative Area)
nmol of GSH-CNDB complex mg protein-1min-1
[TBARS] nmol MDA mg protein -1
a
b
ab
*
*
Transport without anaesthetics
Transport with extract of S acmella
Transport with EO of N. grandiflora
Non-transported fish kept in normoxia
Transport time (hours)

Table 1. Anaesthesia and recovery stages in fish.

Stages	Behaviour
Anaesthesia	
A1	Agitation
A2	Loss of equilibrium and erratic swimming
A3	Minimum opercular beating and loss of reaction to tail pinch stimulus (anaesthesia)
Recovery	
R1	Erratic swimming and recovery of equilibrium
R2	Regular opercular beating and normal swimming

Modified from Park *et al.*, 2008.

Table 2. Anaesthesia induction and recovery times (in seconds ± SD) in juvenile Tambaqui, *Colossoma macropomum* exposed to five concentrations of EO of *Nectandra grandiflora* and extract of jambu *Spilanthes acmella*.

	Concentration	*Induction (s)*			*Recovery (s)*	
N. grandiflora	µL L^{-1}	*stage A1*	*stage A2*	*stage A3*	*stage R1*	*stage R2*
	25	-	34 ± 14c	-	-	84 ± 27b
	50	-	35 ± 20c	-	-	56 ± 29b
	100	-	33 ± 11c	-	-	168 ± 103ab
	200	-	186 ± 23b	1321 ± 198	211 ± 96a	269 ± 161a
	300	-	880 ± 365a	1218 ± 407	70 ± 41b	324 ± 187a
	Equations	-	$y = 110.11x^2 - 476.43x + 451.78$ $r^2 = 0.94$	-	-	$y = 66.71x - 25.477$ $r^2 = 0.90$
S. acmella	mg L^{-1}	*stage A1*	*stage A2*	*stage A3*	*stage R1*	*stage R2*
	5	104 ± 41a	224 ± 96a	408 ± 35a	54 ± 44a	264 ± 133ab
	10	59 ± 15b	103 ± 37b	182 ± 36b	63 ± 32a	171 ± 70b
	15	50 ± 17b	84 ± 28b	166 ± 30bc	50 ± 21ab	230 ± 50ab
	20	35 ± 16b	67 ± 22b	143 ± 15c	25 ± 14b	299 ± 99a
	25	-	26 ±15b	144 ± 63bc	52 ± 31ab	372 ± 137a
	Equations	-	$y = -114.1\ln(x) + 393.64$ $r^2 = 0.94$	$y = 1.2747x^2 - 49.569x + 601.56$ $r^2 = 0.91$	-	$y = 0.9809x^2 - 22.556x + 335.46$ $r^2 = 0.90$

Times to reach subsequent stages during induction or recovery are cumulative.

Different letters in columns indicate statistical differences between concentrations of *N. grandiflora* or *S. acmella* by Tukey test at 5% of probability, n = 10

Table 3. Water quality parameters (Mean ± SD) in the water of tambaqui juveniles submitted to different times of transport with essential oil of *N. grandiflora* and extract of jambu *Spilanthes acmella*.

			Without compound	With *N. grandiflora*	With *S. acmella*
pH	BT		7.4 ± 0.6		
	AT	2 h	6.7 ± 0.03a	6.8 ± 0.08a	6.9 ± 0.21a
		6 h	6.5 ± 0.02ab*	6.5 ± 0.13b*	6.5 ± 0.07b*
		10 h	6.4 ± 0.01b*	6.3 ± 0.05b*	6.3 ± 0.07b*
Alkalinity (mg $CaCO_3$ L^{-1})	BT		65.0 ± 5.0		
	AT	2 h	71.7 ± 5.8	73.3 ± 7.6	76.7 ± 2.9
		6 h	78.3 ± 2.9*	75.0 ± 5.0	73.3 ± 7.6
		10 h	81.7 ± 2.9*	78.3 ± 2.9*	73.3 ± 5.8
T ºC	BT		26.5 ± 0.5		
	AT	2 h	25.4 ± 0.5a	24.9 ± 1.5a	24.0 ± 0.2*
		6 h	24.4 ± 0.2a*	24.2 ± 0.1ab*	24.3 ± 0.3*
		10 h	22.1 ± 0.4b*	22.7 ± 0.3b*	23.5 ± 0.2*
DO (mg L^{-1})	BT		8.6 ± 0.4		
	AT	2 h	19.6 ± 2.9A*	20.2 ± 3.5A*	9.2 ± 0.8Bb
		6 h	18.5 ± 1.1*	16.9 ± 2.5*	16.8 ± 2.0a*
		10 h	20.3 ± 2.8*	17.6 ± 3.2*	14.3 ± 2.1ab*
Total ammonia (mg L^{-1})	BT		0.6±0.1		
	AT	2 h	3.4 ± 0.3*	3.4 ± 0.2*	3.3 ± 0.5*
		6 h	3.5 ± 0.2*	3.5 ± 0.3*	3.5 ± 0.1*
		10 h	3.6 ± 0.1*	3.5 ± 0.2*	3.5 ± 0.3*

Different lower case letters in columns indicate statistical differences over time of transport and different upper case letters in row indicate statistical differences between transports with or without compounds within the same time of transport by two-way Anova and Tukey test at 5% of probability. * Indicates differences between water parameters before transport (BT) and after transport (AT) by repeated measures Anova and Dunnet test at 5% of probability.

Table 4. Induction and recovery times (sec.) in tambaqui, *Colossoma macropomum* with different anaesthetics.

Anaesthetic	Recommended [ ]* ($mg\ L^{-1}$)	Induction (s)	Recovery (s)	Authors
Benzocaine	100 – 150	103 – 163	275 – 532	Gomes *et al.* (2001)
Menthol+	150	130	656	Façanha and Gomes (2005)
Eugenol+	65	152	410	Roubach *et al.* (2005)

* Concentrations necessary to promote fast and deep anaesthesia (corresponding to stage A3).

+ Ethanolic extracts were prediluted in ethanolic vehicle prior to the tests.

CAPÍTULO 4

Transport of juvenile tambaqui *Colossoma macropomum* with new plant extracts and tricaine (MS-222): effects on secondary stress response

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Abstract

The objective of this study was investigate the effect of transport on secondary stress responses in blood of juvenile tambaqui transported with extract of *Spilantes acmella*, essential oil (EO) of *Nectandra grandiflora* and tricaine (TMS®). Fish were placed in plastic bags and transported in hyperoxic conditions for 2, 6 and 10 h with or without anaesthetics. Glycemia, ionic concentration (K^+, Ca^{++}, Na^+) and osmolality, haematocrit (Ht) and haemoglobin concentration (Hb), partial pressure of gases ($pCO2$ and pO_2), pH and bicarbonate concentration (HCO_3^-) were investigated in whole blood collected from caudal vasculature before and after transport. No mortalities were observed at the end of transports, irrespective of duration or the presence of anaesthetics added to the water. Attenuation of secondary stress response was observed with the use of EO of *N. grandiflora* after 6 h of transport based on lower blood glucose levels and no alteration of blood Na^+ concentrations at any transport time compared to reference values of non-transported fish. Furthermore, normoxic levels in the water were still maintained after transport for 10 h with EO of *N. grandiflora* compared to water of non-transported fish. Based on these findings, the use of EO of *N. grandiflora* at the sedation concentration of 30 μL L^{-1} is recommended for this species since it provides a better physiological balance at the end of transport compared to fish transported in anaesthetic-free water. No advantages in terms of secondary stress responses were observed with the use of sedation concentrations of extract of *S. acmella* or TMS® after transport of juvenile tambaqui for up to 10 h.

Introduction

Transport of live fish has increased worldwide as a result of a thriving aquaculture industry (Stieglitz et al., 2012). During the procedure, significant changes in water quality may occur and therefore it acts as a potential stressor that adversely affects the physiology and welfare of fish (Iversen et al., 2009; King, 2009).

Distress during transportation activates the pituitary–interrenal axis in the neuro-endocrine system resulting in release of hormones cortisol and catecholamine as primary stress responses (Barton, 2002). Persistent perturbation elicits secondary stress

response in fish, such as alterations of glycemia and osmoregulation (Carmichael et al., 1983).

The use of anaesthetics during transportation of juvenile fish is frequently used to reduce stress (Ross & Ross, 2008). Fish transported under sedation can maintain normal posture and active opercular movement, but is less excitable. That condition reduces the magnitude and duration of the physiological response (Davis & Griffin, 2004) and therefore mortalities during and post-transport can be minimized.

A number of plant extracts has been investigated and recommended as additives for the transport of live fish, such as: eugenol (Cunha et al., 2010), menthol (Façanha & Gomes, 2005), essential oils of *Eugenia caryophyllata* and *E. aromatica* (Inoue et al., 2003; Bressler & Ron, 2004), *Lippia alba* (Cunha et al., 2011), *Melaleuca alternifolia* (Hajek, 2011), and *Ocimum gratissimum* (Silva et al., 2012).

The Genus *Nectandra* includes approximately 120 known species (Van der Werff & Richter, 1996) and is widely distributed in Amazon and Atlantic forests (Baitello et al., 2003). In Latin America these plants have been used as anti-rheumatic, analgesic (Santos Filho & Gilbert, 1975), sedative (Alves et al., 2008), anti-inflammatory (da Silva et al., 2004), antioxidant (Garcez et al., 2009) and as anaesthetic agents (Tondolo et al., 2013).

Nectandra grandiflora Nees is popularly known as "canela-amarela" and previous work has reported antioxidant properties from crude extracts of the leaves (Ribeiro et al., 2005). Essential oil of *N. grandiflora* also proved to be effective to promote deep anaesthesia in juvenile tambaqui and sedation concentrations added to the transport water enhanced defense against reactive oxygen species mainly in muscle and gills of juveniles submitted to transports of 6 and 10 h in the presence thereof (Barbas et al., *non-published data*).

The Genus *Spilanthes* comprises about 60 species of plants, which are widely distributed in tropical and subtropical regions of Africa, America, Borneo, India and Sri Lanka (Chung et al., 2008; Tiwari et al., 2011). The species *Spilanthes acmella* var oleracea, most commonly known as jambu, Pará or Brazil cress is a plant original from the tropics of Asia and South America (Chung et al., 2008; Prachayasittikul et al., 2013). Previous studies have assessed *S. acmella* extract bioactivity as analgesic and local anaesthetic in rats and mice (Ansari et al., 1988; Nomura et al., 2013). The

presence of N-alkylamides in jambu, mainly "spilanthol" (N-isobutyl-2E,6Z,8E-decatrienamide) suggests that the therapeutic effect observed is a result of its marked anaesthetic activity (Nomura et al., 2013).

Tricaine methane-sulphonate (TMS®), $C_9H_{11}O_2N + CH_3SO_3H$, also known as MS-222 is used for anaesthesia of ectotherms worldwide. It has been approved by the US Food and Drug Administration for use on fish destined for human consumption in the USA. It is also registered for veterinary use in the UK, Canada, Italy, Spain and Norway (Sneddon, 2012; Popovic et al., 2012). Other products such as benzodiazepines, isoeugenol, halothane, lidocaine, benzocaine, ketamine, medetomidine, propofol and carbon dioxide are also often used to induce anaesthesia/sedation in fish (Ross & Ross 2008; Neiffer & Stamper, 2009; Weber 3rd et al., 2009).

Tambaqui *Colossoma macropomum* is an important aquaculture species in the north of Latin America (Araújo-lima & Goulding, 1997; Sevilla & Günther, 2000). It is well adapted in captivity conditions, easily accepting artificial diets and presents good growth rates and feed conversion (Merola & Cantelmo, 1987). Few investigations have been conducted to evaluate the impacts of transport with anaesthetics/sedatives on physiological stress in this species (Gomes et al., 2001; Façanha & Gomes 2005; Gomes et al., 2006).

Proposals of new anaesthetics, coupled with the characterization of the physiological response during and post-transport of the different fish species are necessary to provide aquaculturists with alternative, safe and/or feasible products to be used in fish transportation. To date, there is no information on the impacts of using extract of *S. acmella*, essential oil of *N. grandiflora* and tricaine for the alleviation of secondary stress response in juvenile tambaqui post-transport.

Thus, the objective of the this study was to investigate secondary stress responses in whole blood of juvenile tambaqui *C. macropomum* submitted to transport with extract of *S. acmella*, essential oil of *N. grandiflora* and tricaine for up to 10 h.

Materials and methods

Anaesthetic compounds

Waxes or essential oils, such as those obtained from *S. acmella* and *N. grandiflora* respectively, are poorly diluted in water and therefore required pre-dilution in ethanolic vehicle before using in transport water.

Samples of the extracts of the flowers of *Spilanthes acmella* var *oleracea* (L.) were obtained by fractionated supercritical fluid extraction methodology with the use of CO_2 [SFE(CO_2)] as described by Dias et al. (2012). On the day of the trials, the stock solution of *S. acmella* extract was prepared by weighing and diluting the jambu flowers extract in ethanol (96%) yielding a 2.24 mg mL^{-1} solution which was stored in an amber glass bottle at 4°C until its use.

The essentail oil of *N. grandiflora* was extracted by hydrodistillation using a Clevenger type apparatus for 3 h (Sartor, 2009). A pool from three essential oil samples, obtained from the leaves of the three specimens was prepared. On the day of the trials, the essential oil pool was pre-diluted in ethanol (96%) at a ratio of 1:10, (1 mL of EO diluted in 10 mL of ethanol) so that each mL of the solution contained 100 μL of EO. The stock solution was stored at 4 ºC in amber glass bottles until biological tests. For more details on the extraction methodology and the EO components see Silva (2013).

Before experiments, tricaine (TMS®) (Sigma Chemical E10521 - St Louis, MO, USA) was solubilized in deionized water and buffered with sodium bicarbonate, using a ratio of 1:1 (sodium bicarbonate : tricaine methanesulfonate powder), providing a final concentration of 10 mg mL^{-1} (pH 7.0) from which aliquots were taken and used in the water of the trials.

Experimental animals

Juvenile tambaqui (65.74 ± 15.35 g and 16.07 ± 1.02 cm) were obtained from a fish farm in the State of Amazonas, northern Brazil. The fish were maintained in a recirculation system for a 15-day acclimation period, in tanks with 200 L of fresh water, stocking density ~ 5 g L^{-1} and constantly aerated. Fish were fed twice a day with

commercial feed (28% crude protein) at 2% of biomass during the acclimation period. Thirty minutes after feeding, the faeces and food remains were siphoned out and new water was added to the tank to make up the volume.

Water Quality

In the acclimation tanks the water quality parameters (mean ± SD) were maintained as follows: Dissolved Oxygen (DO) 6.40 ± 0.37 mg L^{-1} and temperature 26.0 ± 0.69 °C were measured using an oxygen meter (Yellow Springs Instruments, Yellow Springs, OH, USA); pH 6.8 ± 0.2 was determined with a Five Easy FE20, Switzerland. Total ammonia nitrogen (TAN) 0.80 ± 0.08 mg L^{-1} NH_4^+ + NH_3^- - N was quantified according to Unesco (1983) and nitrite 0.03 ± 0.01 mg L^{-1} was determined according to (Bendschneider & Robinson 1952). Total alkalinity was evaluated by titration in accordance with Eaton et al. (2005) guidelines and was 50.7 ± 0.9 mg L^{-1}.

Transport and evaluation of physiological indicators

Concentrations ranging from 8 up to 15% of the effective anaesthetic concentrations capable of promoting deep anaesthesia (Barbas et al., *non-published data*) were used herein to induce slight anaesthesia in this species, clinically characterized by lethargy and reduced response to external stimuli. These concentrations corresponded to 2 mg L^{-1}, 30 μL L^{-1} and 20 mg L^{-1} of extract of *S. acmella*, EO of *N. grandiflora* and TMS®, respectively.

Juveniles were weighed and placed in plastic bags (30 L) with 5 L of water that were inflated with pure oxygen (3 parts of oxygen : 1 part of water) and tied with rubber bands. The load density was set at approximately 160 g L^{-1} (12 specimens per bag and bags were in triplicate per group), which corresponds to the density used in commercialization of this species in the Amazon region. Fish were transported on paved road for 2, 6 and 10 h with or without anaesthetics and the experimental design was set as follows:

i. Two, six and ten hours of transport in anaesthetic-free water (with and without ethanol);

ii. Two, six and ten hours of transport with extract of *S. acmella*,;

iii. Two, six and ten hours of transport with EO of *N. grandiflora*;

iv. Two, six and ten hours of transport with TMS®.

At the end of each period (2, 6 and 10 h), juveniles were blood sampled (2 or 3 per replicate) and blood glucose, ions (Na^+ K^+ and Ca^{++}), Htc, Hb, partial pressure of CO_2 ($pCO2$), partial pressure of O_2 (pO_2), pH and bicarbonate concentration (HCO_3^-) were determined with a portable i-STAT® clinical analyser using CG8+ cartridges (Abbott laboratories, Chicago, IL, USA). The values obtained for $pCO2$, pO_2, pH and HCO_3^- were corrected for the water temperature according to manufacturer's instructions and also as described by Hanley et al. (2010). Blood osmolality was determined immediately after collection using a vapour-pressure osmometer (Vapro 5520; Wescor, Inc.; Logan, Utah, USA).

Water parameters: alkalinity, dissolved oxygen, pH, and total ammonia were determined at the beginning (0 h) and at the end of each period (2, 6 and 10 h).

This study was approved by the Ethics Committee on Animal Experimentation of the Universidade Federal do Rio Grande - FURG.

Statistical analysis

All data are presented as mean ± SD. To verify the homogeneity of variances and normality, data were submitted to Levene and Kolmogorov-Smirnov tests, respectively. The results obtained for glucose, HCO_3^- concentration and $pCO2$ were log transformed previously to statistical analysis. When assumptions were satisfied, a two-way Anova was used and when statistical differences were detected Tukey-kramer post-hoc test was used for pair-wise comparisons. Osmolality data were submitted to non-parametric Kruskal Wallis test. Water quality parameters before and after transport were submitted to repeated measures Anova and Dunnet test. The minimum significance level was set at $p<0.05$ in all cases.

Results

No mortalities were observed at the end of any transport time, irrespective of the presence of anaesthetics added to the water.

Secondary stress response

Secondary stress response in whole blood of tambaqui post-transport is presented in Table 2.

Fish transported for 6 h in the presence of EO of *N. grandiflora* showed decreased glucose levels ($p<0.05$) compared to controls (CT) transported for the same length of time. Glucose levels were significantly increased ($p<0.05$) during transport for most of the treatments compared to non-transported fish (NTF).

Significant differences were only observed in osmolality as a function of time for transports with *S. acmella* extract and EO of *N. grandiflora*, whereupon osmolality was lower ($p<0.05$) after transport for 2 or 10 h compared to fish transported for 6 h in the first case and significant differences were observed in the latter with lower ($p<0.05$) osmolality observed after 2 h compared to fish transported for 6 h. Nevertheless, no differences ($p>0.05$) were observed in osmolality regardless of the presence of anaesthetics in the water.

A lower concentration of K^+ ($p<0.05$) was observed in fish after 10 h of transport with TMS® compared to values of NTF. Lower Na^+ concentrations ($p<0.05$) were observed in fish transported with extract of *S. acmella* ($p<0.05$) compared to reference values of NTF. Sodium concentrations were also lower after 10 h transport in CT and from 6 h transport and forward in blood of fish transported with TMS® compared to values of NTF.

Haematological variables Hct and Hb concentration showed the same response pattern in transports with TMS®, with increased levels ($p<0.05$) after 10 h of transport compared to fish transported for 2 h with the same anaesthetic and also higher compared to NTF.

Partial pressure of carbon dioxide ($pCO2$) increased ($p<0.05$) over time both in controls and in fish transported with anaesthetics. On the other hand, fish transported

for 6 h with EO of *N. grandiflora* or TMS® showed decreased ($p<0.05$) *p*CO2 in blood compared to CT transported for the same length of time.

Bicarbonate concentration (HCO_3^-) was increased ($p<0.05$) after transports for 6 h for all treatments compared to transports of 2 h, however, no differences ($p>0.05$) were observed between fish transported with or without anaesthetics within each transport time, except for a lower concentration of HCO_3^- in transport with *S. acmella* extract for 2 h compared to CT transported for the same length of time.

Both *p*CO2 and HCO_3^- concentration were consistently increased ($p<0.05$) in the blood of all specimens submitted to transport with or without anaesthetics compared to NTF.

A decrease ($p<0.05$) in pO_2 was observed in fish transported with extract of *S. acmella* for 6 h and forward compared to values of fish transported for 2 hours with the same anaesthetic.

Blood pH was not affected ($p>0.05$) over time regardless of the presence of anaesthetics. A significantly lower ($p<0.05$) blood pH was only observed after 6 h of transport in CT compared to NTF.

Water quality

Parameters of water quality were measured before and after transports and are provided in Table 3.

Lower ($p<0.05$) pH and temperature were observed in the water after transport, regardless of transport duration or the presence of anaesthetics compared to water before transport.

Most of the treatments showed no significant alterations ($p>0.05$) in water alkalinity after transport compared to water before transport, except for transports after 6 h without anaesthetics and after 10 h of transport in the presence of *S. acmella* extract in which alkalinity was increased and reduced ($p<0.05$), respectively.

Dissolved oxygen levels gradually decreased ($p<0.05$) over time as in the cases of transport with ethanol and *S. acmella* extract. Moreover, in transports without anaesthetics and with TMS® for 6 and 10 h, dissolved oxygen (DO) concentrations in the water were significantly lower ($p<0.05$) compared to transports for 2 h within their

respective groups. On the other hand, water added by EO of *N. grandiflora* did not show significant changes ($p>0.05$) in DO over time, whereas after 2 h of transport with this oil, DO concentrations were significantly ($p<0.05$) decreased compared to the water of fish transported without anaesthetic for the same time. Yet, after 10 h of transport with EO of *N. grandiflora*, DO was higher in the water compared to the water from bags transported with ethanol or extract of *S. acmella*.

Furthermore, DO levels were significantly higher ($p<0.05$) after transports for 2 h for all groups and significantly reduced ($p<0.05$) after 6 h of transport without anaesthetics and 10 h of transport in the presence of ethanol, *S. acmella* extract and TMS® compared to water before transport.

Total ammonia concentration was significantly increased ($p<0.05$) after transport for all treatments compared to water of NTF.

Discussion

The acidification of water after transport is mainly a consequence of CO_2 respiration and these changes in water pH may have an effect on blood acid-base balance (Treasurer, 2012). However, the water pH at the end of the transports (6.2 - 6.8) was within the range observed in natural occurrence sites of tambaqui (Wood et al., 1998) which indicates that pH post-transport was not a threat. In contrast to our findings, Gomes et al. (2006) did not observe changes in the water pH of juvenile tambaqui submitted to transport of up to 24h.

Temperature was decreased after transport for all groups and although transport bags were sealed inside Styrofoam boxes prior to transports, it was possibly affected by relatively low environmental temperature (12 to 15 ºC) on the day of the trials. Nevertheless, since no significant variations were observed for pH or temperature among transported groups with or without anaesthetics and also over time, differences in physiology were not triggered by those water quality variables.

Although DO concentration was not measured in bags immediately after the injection of pure oxygen, the methodology for inflating the bags was identical (O_2/H_2O ratio of 3:1) among treatments and presumably, DO concentrations were in similar conditions at the beginning of transports. As expected, DO concentration decreased over

time in the water, except for transports with EO of *N. grandiflora*, in which this compound seemed to have played an important role in maintaining good oxygen availability even after 10 h of transport whereby levels of oxygen were still similar to those of normoxic water before transport.

Concentration of total ammonia was higher in the water after transport compared to water of non-transported fish, however, total ammonia was not increased over time for transported fish and was not ameliorated by the use of anaesthetics during transport. Tambaqui is an extremely resistant species to the toxic effect of NH_3 (Ismiño-Orbe, 1997). Since water was slightly acidotic (mean pH 6.53) in the bags after transport and mean temperature was constant (24.2 ºC) post-transport, ammonia toxicity fraction was only about 0.1% of total ammonia, as can be estimated from published tables (Boyd, 1982). As a result, ammonia toxicity was extremely low.

The use of portable chemical analyzers to evaluate physiological status including calculations for blood gas tension, has been described for several species of fishes (Jacobs et al., 1993; Pidetcha et al., 2000; Sladky et al., 2001; Harrenstien et al., 2005; Kristensen et al., 2010).

Tambaqui in this study were clearly affected by pre-transport handling and the transport procedure *per se* as one can see from the significant changes occurred mainly in glycemia, blood CO_2 tension, Na^+ and HCO_3^- concentrations for the majority of transported fish compared to NTF. Nevertheless, secondary stress responses among transported fish were in most cases undistinguishable regardless of its duration or the presence of anaesthetics added to the water.

In the case of acute stress, elevations in plasma glucose are likely maintained by catecholamine-mediated glycogenolysis (Mommsen et al., 1999; Begg & Pankhurst, 2004; Liebert & Schreck 2006). Glycemia level is frequently reported as a reliable stress indicator for fish (Wedemeyer et al, 1990; Svobodová et al, 1999; Iversen et al., 2005; Gomes et al., 2003; Iversen et al., 2009; Oyoo-Okoth et al., 2011). Although significant increases in blood glucose were observed in transported fish compared to NTF, glucose release to circulation was generally neither intensified over transport time nor attenuated by the use of the anaesthetics. The exception was a decreased glucose level observed after 6 h of transport in the water added by EO of *N. grandiflora* compared to CT transported for the same time. Yet, after 6 h of transport with EO of *N.*

grandiflora glycemia was maintained at the same level of NTF. The use of this EO provides attenuation of the acute stress response after 6 h transport and therefore fish are expected to better cope with the effects of stress brought about by the transport procedure.

Tambaqui is an omnivorous freshwater fish and glucose concentration analyses, even by means of portable instruments, have been already validated (Gomes et al., 2005) and glycemia is regarded as a reliable parameter to evaluate acute stress responses in blood and plasma of this species (Gomes et al., 2003; Inoue et al., 2011).

Despite some significant reductions in blood ions, mainly in Na^+ concentrations observed after 10 h transport, regardless of the presence of anaesthetics and more markedly for transports with extracts of *S. acmella* compared to NTF, no major osmoregulatory disturbances were observed after transport with or without anaesthetics, based on ionic concentrations and blood osmolality. Increased branchial ion losses during stress are not always reflected by a reduction of plasma osmolality (Wendelaar Bonga, 1997). It has been reported that during acute stress, plasma water can move out of the circulation and into the tissues (Okimoto et al., 1994), which in turn could mask the osmoregulatory status of freshwater fish with an unchanged or even increased osmolality and electrolyte concentrations in plasma after a distressing event. Moreover, one cannot rule out that mild electrolyte alterations may occur in fish due to normal ion diffusion through the gills as a consequence of environmental manipulations (Stoskopf, 1993).

Haematocrit and Hb concentration showed the same response pattern after transport in all treatments and were virtually unchanged. In acute stress challenges, haematological stress responses are rarely reflected in those parameters, being more responsive when under chronic stress conditions (Morales et al., 2005).

The pO_2 parameter showed more variation than the other variables examined, which was rather expected because there is a potential for collection of mixed venous-arterial blood samples from the caudal vasculature. This methodological limitation was also reported by Sladky et al. (2001) when using identical analytical procedures to calculate pCO_2 in blood of red pacu, *Piaractus brachypomus* collected in a similar fashion.

Several authors have reported hypercapnia as a direct consequence of increased

CO_2 levels in the water (Hosfeld et al., 2008; Tang et al., 2009; Petochi et al., 2011). Although CO_2 concentrations were not measured in the water in this study, it is plausible that increasing concentrations in the water over time were directly contributing to the gradual increase of pCO_2 in blood over time in transported fish.

Acid–base relationship can be affected by hypercapnia (Crocker & Cech, 1998) with a consequent acidification of blood (Hayashi et al., 2004a, 2004b). No substantial alteration of acid-base balance was observed in transported fish regardless of transport time or the presence of anaesthetics in the water. This steady blood pH condition may have played an important role in minimizing further ion losses since it is known that control of blood or plasma pH in aquatic animals is intimately associated with ionic regulation at the gills (Walsh & Milligan, 1989). Presumably, blood chemistry in tambaqui can accommodate for these changes, as it has been described for yellowtail kingfish *Seriola lalandi*, (Moran et al., 2008).

Compensatory mechanisms involving ion regulation can be activated in response to blood acidosis such as elevation of plasma concentration of HCO_3^- (Wood & Jackson, 1980; Heisler, 1993) in response to the catalytic action of carbonic anhydrase in the gills and in erythrocytes (Tufts & Perry, 1998), accompanied by the excretion of protons (H^+) by an Na^+/H^+ antiporter in erythrocytes or through a V-ATPase in the gills and the excretion of HCO_3^- through an HCO_3^-/Cl^- antiporter (Marshall & Grosell, 2006). Therefore, a rise in pCO_2 is usually associated with a transient acidosis and a pronounced increase in plasma concentrations of HCO_3^- (Heisler, 1984; Perry & Gilmour, 2006), such as the significant elevations in HCO_3^- concentrations observed in the blood of transported *vs.* NTF fish and over time after 6 h transport and forward compared to fish transported for 2 h with or without anaesthetics. Juvenile tambaqui showed compensatory capacity against hypercapnic stress by increasing HCO_3^- in blood regardless of the presence of anaesthetics in the water.

In summary, fish producers depend on optimal techniques to ensure better profit, therefore, costs in fish culture must be minimized. No advantages were observed with the use of extracts of *S. acmella* or TMS® in the water for any transport time in this study. Similar results have been reported and the use of additives such as benzocaine at concentrations of 10 up to 30 mg L^{-1} did not improve juvenile tambaqui survival after transports for 3 up to 24h compared to controls (Gomes et al., 2006). On the other hand,

an interesting attenuation of secondary stress response was observed with the use of EO of *N. grandiflora* after 6 h of transport based on lower blood glucose levels and unchanged Na^+ concentrations at any transport time compared to non-transported fish. Furthermore, higher levels of oxygen in the water were observed after transport for 10 h with EO of *N. grandiflora* compared to ethanol control. Thus, one can expect that the re-establishment of homeostasis can be enhanced during recovery after transport with this oil. Future investigations should be conducted on tambaqui as well as on other Amazon species with different sedation concentrations of extract of *S. acmella* and TMS®, other than those used in this study, to prospect potential benefits of these compounds based on the attenuation of stress response during and post-transport.

In conclusion, sedation concentrations of extract of jambu, *Spilanthes acmella* and TMS® did not imply significant advantages to alleviate secondary physiological stress response, whereas the use of EO of *N. grandiflora* added to the water at the concentration of 30 μL L^{-1} attenuated secondary stress response in juvenile tambaqui after 6 h of transport in plastic bags.

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Table 1 - Water quality parameters (Mean±SD) in the water of tambaqui juveniles submitted to different times of transport (2, 6 and 10 hours) with essential oil of *N. grandiflora* (30 µL L^{-1}), extract of *Spilanthes acmella* (2 mg L^{-1}) and TMS® (20 mg L^{-1})

			Without anaesthetics	With Ethanol	With *S. Acmella*	With *N. grandiflora*	With TMS®
pH	BT		7.40±0.02				
	AT	2 h	6.88±0.06*	6.83±0.02*	6.60±0.06*	6.74±0.20*	6.85±0.04*
		6 h	6.49±0.01*	6.57±0.04*	6.28±0.04*	6.52±0.06*	6.52±0.13*
		10 h	6.40±0.02*	6.37±0.04*	6.22±0.03*	6.36±0.07*	6.42±0.07*
Alkalinity (mg $CaCO_3$ L^{-1})	BT		63.0±2.8				
	AT	2 h	65.0±1.0	65±1.0	72.0±2.8	73.0±5.7	68.0±2.8
		6 h	78.0±2.5*	75±1.0	57.0±2.8	63.0±2.8	67.0±7.6
		10 h	73.0±2.5	65±1.0	50.0±5.0*	57.0±2.8	63.0±12.5
T (ºC)	BT		26.8±0.3				
	AT	2 h	24.8±0.1*	24.8±0.1*	24.2±0.8*	23.8±0.1*	23.7±0.2*
		6 h	24.2±0.2*	23.8±0.1*	24.6±0.2*	24.4±0.2*	24.4±0.3*
		10 h	24.1±0.1*	23.7±0.1*	24.6±0.1*	24.2±0.1*	24.2±0.1*
DO (mg L^{-1})	BT		8.47±0.15				
	AT	2 h	21.13±2.53aA*	17.19±0.78aAB*	17.51±3.51aAB*	14.66±1.16B*	16.52±3.07aAB*
		6 h	3.55±0.65bB*	10.65±1.75bA	9.50±3.36bAB	9.65±1.25AB	8.90±2.64bAB
		10 h	4.58±1.83bAB*	2.04±0.22cB*	3.10±1.42cAB*	8.61±0.94A	3.54±1.75bAB*
Total ammonia (mg L^{-1})	BT		0.72±0.1				
	AT	2 h	4.8±0.9*	5.2±0.9*	4.8±0.8*	4.4±0.7*	5.2±0.9*
		6 h	6.5±0.4*	7.5±0.6*	6.4±0.5*	4.5±0.9*	6.4±0.5*
		10 h	7.6±0.9*	7.5±0.8*	7.0±0.3*	6.4±0.8*	6.5±0.7*

When present, different lower case letters in columns indicate statistical differences of water quality parameters between transport time and different upper case letters in row indicate statistical differences of water quality parameters among transports with ethanol, anaesthetics or without anaesthetics within the same transport time by two-way Anova and Tukey test ($p<0.05$). * Indicates differences between initial water quality conditions before transport (BT) and after transport (AT) by repeated measures Anova and Dunnet test ($p<0.05$).

Table 2. Glycemia, ionic concentration (K^+, Ca^{++}, Na^+) and osmolality, haematological variables (Ht and Hb), partial pressure of gases ($pCO2$ and pO_2), pH and bicarbonate concentration (HCO_3^-) in whole blood of juvenile tambaqui, *C. macropomum* transported for 2, 6 and 10 hours in the absence or presence of plant extracts (*S. acmella* and *N. grandiflora*) and TMS® (MS-222). Values are expressed as means ± SD.

	Glucose (mg dL^{-1})	**K^+ (mmol L^{-1})**	**Ca^{++} (mmol L^{-1})**	**Na^+ (mmol L^{-1})**	**Osmolality (mOsm kg^{-1})**	**Ht (%)**	**Hb (g dL^{-1})**	***p*CO_2 (mm Hg)**	***p*O_2 (mm Hg)**	**pH**	**HCO_3^- (mmol L^{-1})**
NTF	54.11±3.79	3.13±0.36	1.31±0.11	149.44±1.24	294.44 ± 5.13	22.22±2.82	7.57±0.97	7.74±1.08	8.02±4.00	7.57±0.09	6.52±0.83
CT											
2 h	169.50±31.54*	3.10±0.46	1.40±0.11	140.50±3.45	305±6	21.83±5.42	8.00±0.86	16.49±0.88b*	9.34±3.73	7.54±0.03	14.21±0.98b*
6 h	171.67±44.50*	2.54±0.30	0.98±0.17	138.67±3.67	298±10	26.83±3.31	9.13±1.11	31.71±1.22a*	7.79±4.35	7.42±0.04*	20.77±1.22a*
10 h	131.5±39.48*	2.50±0.32	1.23±0.28	138.00±0.71*	286±7	24.40±6.62	8.30±2.26	32.70±1.25a*	7.57±2.90	7.51±0.03	26.38±1.49a*
With *S. acmella*											
2 h	132.44±46.14*	2.92±0.49	1.28±0.26	137.33±4.74*	290±13b	25.22±4.71	8.57±1.61	14.98±1.22c*	15.69±10.36a	7.49±0.05	11.16±1.80b*+
6 h	114.77±17.71*	2.80±0.47	1.18±0.19	138.55±2.18*	315±5a	25.00±3.27	8.50±1.11	29.20±2.27b*	7.70±3.70b	7.48±0.04	22.37±2.82a*
10 h	121.67±31.74*	2.58±0.45	1.16±0.26	136.78±2.22*	283±10b	28.44±4.59	9.68±1.58	35.29±2.69a*	5.33±2.58b	7.48±0.04	26.29±2.21a*
With *N. grandiflora*											
2 h	110.89±20.42*	2.64±0.42	1.39±0.17	140.00±1.94	289±8b	22.22±1.72	7.56±0.59	16.27±1.22c*	12.16±5.72	7.55±0.05	14.16±1.29b*
6 h	104.22±19.89+	2.50±0.30	1.17±0.20	141.56±2.65	316±6a	22.11±2.47	7.52±0.82	25.47±1.57b*+	9.23±3.40	7.53±0.06	21.43±2.79a*
10 h	94.78±24.05	2.64±0.37	1.28±0.23	141.22±2.28	303±16ab	24.44±4.13	8.31±1.41	30.22±1.79a*	10.58±3.93	7.50±0.05	23.89±2.82a*
With TMS®											
2 h	120.89±18.58*	2.89±0.49	1.37±0.22	140.33±2.60	308±3	22.67±2.29b	7.72±0.77b	16.20±1.45c*	13.20±5.99	7.52±0.06	13.18±1.10b*
6 h	152.42±53.43*	2.48±0.21	1.15±0.27	137.11±4.86*	296±20	25.56±3.05ab	8.70±1.03ab	23.97±1.77b*+	7.25±2.54	7.51±0.04	21.63±2.01a*
10 h	124.78±35.93*	2.36±0.34*	1.20±0.25	137.44±3.81*	296±19	30.00±4.00a*	10.19±1.38a*	33.35±4.86a*	6.18±1.83	7.51±0.07	26.78±3.30a*

When present, different letters in columns indicate significant differences among transport times within groups of fish transported with the same anaesthetic or without anaesthetic (CT). + Indicates significantly different from CT (control group transported without anaesthetic for

the same length of time); * indicates significantly different from NTF (non-transported fish from acclimation tanks), n = 9 or 6 (as in the case of CT)

4. CONSIDERAÇÕES FINAIS

Os extrativos vegetais de jambu (*S. acmella*) e canela-amarela (*N. grandiflora*) foram eficazes como anestésicos para peixes e os benefícios da utilização de ambos como produtos antioxidantes foram demonstrados. Como são vegetais abundantes no país, a exploração racional desses recursos é factível.

A tricaína (MS-222) é um dos poucos anestésicos permitidos para uso em organismos aquáticos, sendo esse fármaco regulamentado por agencias governamentais de alguns países na Europa e América do norte. Apesar da relativa dificuldade de aquisição e da não disponibilização da tricaína no mercado nacional, os resultados do presente estudo podem ser decisivos para a regulamentação de seu uso no país, mesmo que outros compostos já estejam sendo frequentemente utilizados, entretanto, sem a devida regulamentação.

Como resultado deste trabalho de Tese também foi apresentado ao Instituto Nacional de Propriedade Industrial (INPI) uma proposta de registro de Patente no país com os dados parciais obtidos e descritos em um dos manuscritos que trata da eficácia do extrato de jambu *S. acmella* como anestésico para juvenis de tambaqui (Capítulo 3). A proposta de Patente intitulada "COMPOSIÇÃO ANESTÉSICA A BASE DE EXTRATO VEGETAL E MÉTODO DE SEDAÇÃO E/OU ANESTESIA DE PEIXES" foi depositada no INPI sob o N.º de processo: **BR 10 2014 012070 0** na Data 20/05/2014.

5. CONCLUSÕES

1) O extrato de jambu, *S. acmella* foi eficaz e seguro para induzir anestesia profunda e recuperação calma em juvenis de tambaqui, *C. macropomum* (46,6 g) em todas as concentrações testadas. Ademais, o uso de 20 mg L^{-1} foi suficiente para induzir à anestesia rápida e profunda (em menos de 3 min) e ocasionou alterações fisiológicas transitórias no sangue durante a recuperação;

2) A tricaína (TMS®) foi eficaz e segura para anestesia de juvenis de tambaqui sendo necessária a concentração de pelo menos 240 mg L^{-1} para indução rápida e profunda

nesta espécie. Não foram observadas vantagens na adição de TMS® à água de transporte para a atenuação das respostas de stress oxidativo nos tecidos com a utilização da concentração de sedação de 20 mg L^{-1};

3) O extrato de jambu, *S. acmella* e o óleo essencial (OE) de *N. grandiflora* mostraram-se anestésicos eficazes para tambaqui (3,3 g), induzindo anestesia profunda e recuperação calma. O uso de 10 mg L^{-1} de extrato de *S. acmella* foi suficiente para induzir à anestesia rápida e recuperação em tempo adequado para juvenis com este peso. Sedação com 1 mg L^{-1} e 30 μ L^{-1} de de extrato de *S. acmella* e OE de *N. grandiflora*, respectivamente, são recomendáveis para o transporte uma vez que esses compostos conferiram proteção contra danos oxidativos, principalmente no músculo e brânquias;

4) As concentrações de sedação de extrato de jambu, *S. acmella* e tricaína adicionadas a água de transporte não implicaram em vantagens significativas para amenizar a resposta secundária de estresse no pós-transporte, enquanto que a utilização de OE de *N. grandiflora* adicionado à água na concentração de 30 mL L^{-1} atenuou essas respostas em juvenis de tambaqui transportados por 6 h em sacos plásticos.