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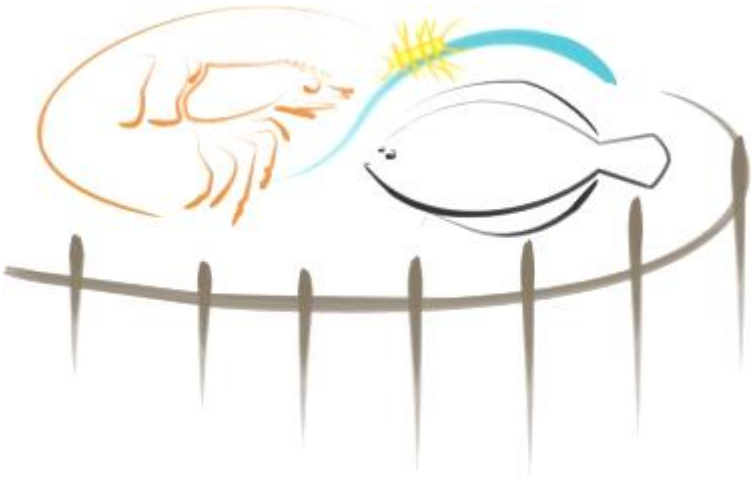

Anestesia e transporte de juvenis de bijupirá *Rachycentron canadum*

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Tese apresentada como parte dos requisitos para a obtenção do grau de Doutora em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande – FURG.

Orientador: Dr. Luís André Sampaio

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SUMÁRIO

DEDICATÓRIA

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RESUMO GERAL

O transporte é uma atividade importante em aquicultura, porém considerada uma das mais estressantes para peixes. Já os anestésicos são utilizados para reduzir as respostas de estresse. Assim, o presente trabalho objetivou avaliar técnicas para melhorar o transporte de juvenis de bijupirá, bem como verificar a eficácia de mentol, MS-222 e eugenol como anestésicos para bijupirá. As técnicas utilizadas para estudar o transporte foram avaliação de diferentes densidades de estocagem (15, 24 e 33 g L^{-1}) com utilização de tampão (1 g $NaHCO_3$ L^{-1}) para manutenção do pH, combinação de diferentes salinidades e temperaturas (S12-T19, S30-T19, S12-T23 e S30-T23) e utilização do anestésico mentol (0, 2,5 e 5,5 mg L^{-1}). Os anestésicos avaliados para práticas de manejo foram o MS-222 (60, 80, 100 e 120 mg L^{-1}), o eugenol (20, 30, 40 e 50 mg L^{-1}) e o mentol (35, 45 e 55 mg L^{-1}). Para os experimentos de transporte foram utilizados bijupirás de ≈ 30 g. No primeiro experimento de transporte, o tampão bicarbonato de sódio foi eficaz em manter o pH da água acima de 7 nas diferentes densidades avaliadas, ocorrendo mortalidade (18,2 %) apenas na densidade 33 g L^{-1}. No segundo experimento de transporte, a combinação entre a salinidade 12 g L^{-1} (ponto isosmótico) com a temperatura 19 °C foi eficiente para o transporte (densidade: 27 g L^{-1}), pois manteve alta a concentração de oxigênio e amenizou a resposta ao estresse. As mortalidades ocorreram apenas para as combinações S30-T19 (1,2 %) e S30-T23 (7,4 %). As mortalidades ocorridas nos experimentos de transporte foram atribuídas principalmente à hipóxia e à hipercapnia. O estudo do mentol no transporte utilizou as técnicas dos experimentos anteriores (1 g $NaHCO_3$ L^{-1} + S12-T19), apesar de não ocorreram mortalidades (densidade: 26 g L^{-1}), o mentol não foi eficaz em reduzir as

respostas de estresse. Em relação à avaliação dos anestésicos, as concentrações 100 mg MS-222 L^{-1}, 30 mg eugenol L^{-1} e 55 mg mentol L^{-1} foram as menores a induzir anestesia em juvenis de bijupirá (≈ 50 g), porém a mesma eficácia não se refletiu na redução de respostas secundárias de estresse, apesar de após 24 h da anestesia os peixes já terem retornado a suas condições normais. Assim, o tamponamento do pH juntamente com emprego de meio isosmótico e temperatura de ≈ 19°C, são condições recomendadas para transportar juvenis de bijupirá (≈ 30 g) a uma densidade de até 27 g L^{-1}. As concentrações 100 mg MS-222 L^{-1}, 30 mg eugenol L^{-1} e 55 mg mentol L^{-1} foram consideradas eficazes para induzir a anestesia em juvenis de bijupirá (≈ 50 g). O mentol não é recomendade pra o transporte de juvenis de bijupirá.

ABSTRACT

Transport is an important activity for aquaculture, although being considered one of the most stressing for fish. Anesthetics are used to reduce stress response. Therefore, this study aimed to evaluate techniques to improve juvenile cobia transport, as well as verify the effectiveness of menthol, MS-222, and eugenol as anesthetics for handling cobia. Transport was studied at different stocking densities (15, 24, and 33 g L^{-1}) with a buffer addition (1 g $NaHCO_3$ L^{-1}) to maintain pH, combination of different salinities and temperatures (S12-T19, S30-T19, S12-T23, and S30-T23) and the use of the anesthetic menthol (0, 2.5 and 5.5 mg L^{-1}). The anesthetics verified for handling practices were MS-222 (60, 80, 100, and 120 mg L^{-1}), eugenol (20, 30, 40, and 50 mg L^{-1}) and menthol (35, 45 and 55 mg L^{-1}). Cobia weighing ≈ 30 g was used for transport experiments. In the first transport experiment, the buffer sodium bicarbonate was efficient in keeping pH above 7 at the different stocking densities evaluated, with mortality (18.2 %) just at 33 g L^{-1}. In the second transport experiment, the combination between salinity 12 g L^{-1} (isosmotic point) with the temperature 19°C was efficient for the transport (density: 27 g L^{-1}), since it kept a higher oxygen concentration and minimized the stress response. Mortalities occurred just at the combinations S30-T19 (1.2 %) and S30-T23 (7.4 %). The mortalities from both experiments were attributed mainly due to hypoxia and hypercapnia. In the third transport experiment, now with menthol addition, techniques from the previous experiments were used (1 g $NaHCO_3$ L^{-1} + S12-T19) and no mortalities were found (density: 26 g L^{-1}), nonetheless menthol was not effective to reduce stress response. Related to the anesthetics evaluation, the concentrations 100 mg MS-222 L^{-1}, 30 mg eugenol L^{-1} and 55 mg menthol L^{-1} were the lowest to induce

anesthesia in juvenile cobia (≈ 50 g), however the same effectiveness was not reflected in the reduction of secondary stress responses, although fish recovered to their normal conditions after 24 h from anesthesia. Therefore, the pH buffering plus the use of isosmotic medium and temperature of ≈ 19°C, are conditions recommended for juvenile cobia (≈ 30 g) transport in densities up to 27 g L^{-1}. Besides that, the concentrations 100 mg MS-222 L^{-1}, 30 mg eugenol L^{-1} and 55 mg menthol L^{-1} were efficient to induce anesthesia in juvenile cobia (≈ 50 g). Menthol is not recommended for juvenile cobia transport.

INTRODUÇÃO GERAL

Bijupirá *Rachycentron canadum*

O bijupirá *Rachycentron canadum* (Linnaeus, 1766) (Figura 1) é o único membro da família Rachycentridae. É um peixe cosmopolita, pelágico e migratório, tendo sua ocorrência em várias regiões ao redor do mundo, em áreas tropicais, subtropicais e em áreas temperadas, em temperaturas que variam de 17 a 32°C (Figura 2). Não é encontrado apenas no Mar Mediterrâneo e na costa leste do Oceano Pacífico (Shaffer e Nakamura, 1989).

Figura 1. Reprodutor de bijupirá (Foto: Luís A. Sampaio).

Figura 2. Distribuição do bijupirá *Rachycentron canadum* (Adaptado de www.fishbase.org por Marcelo Okamoto).

O bijupirá é considerado uma importante espécie para a aquicultura devido a vários fatores: rápido crescimento, podendo alcançar de 4 a 6 kg em um ano; elevada sobrevivência na fase de juvenil e engorda; eficiência alimentar relativamente alta para uma espécie carnívora, com taxas de conversão próximas a 1,5:1; carne de excelente qualidade; bom crescimento em salinidade de até 5 g L^{-1}; desova natural em cativeiro e protocolo de larvicultura já estabelecido (Arnold et al., 2002; Resley et al., 2006; Holt et al., 2007; Webb Jr. et al., 2007; Benetti et al., 2010;).

A estrutura de produção mais utilizada para a produção de bijupirá é tanque-rede. Os principais países produtores são Taiwan, China, Vietnam e Panamá, os quais produziram aproximadamente 40 mil toneladas em 2014 (Benetti et al., 2010; FAO, 2016).

O crescente aumento de sua produção nessas regiões atraiu a atenção de instituições de pesquisa e do setor privado brasileiro, sendo que a partir de 2008 o Ministério da Pesca e Aquicultura (MPA - atualmente extinto) concedeu permissão para a criação de bijupirá em tanques-redes (Sampaio et al., 2011). Devido às suas características ambientais, com grande área apta para a produção, o Brasil possui ótimas condições para a criação de bijupirá, porém ainda enfrenta problemas, devido a produção instável de juvenis, fornecimento de dietas adequadas, problemas relacionados à sanidade e dificuldades no processo de licenciamento ambiental (Cavalli et al., 2011).

Domingues et al. (2014) avaliaram a viabilidade econômica da produção de bijupirá em tanques-redes no litoral de Pernambuco, e concluíram que a atividade só

seria rentável se a produtividade fosse maior ou igual a 10 kg m^{-3} e se o valor de venda fosse de pelo menos R$15,00 kg^{-1}.

Em uma criação particular em tanques-redes instalados próximo à costa no litoral do Rio de Janeiro, o peso médio final dos bijupirás após um ano foi de 4,2 kg, porém peixes com 6 kg também foram observados (Sampaio et al. 2011). Em igual período, Benetti et al. (2010) observaram peso final de 4-6 kg para bijupirás em gaiolas localizadas em Porto Rico e Bahamas.

Há necessidade de um maior conhecimento em relação às respostas adaptativas aos procedimentos rotineiros em aquicultura para o bijupirá, pois eles ainda são pouco estudados. As respostas sobre suas reações fisiológicas à diversos fatores estressantes, comuns ao manejo, sejam eles crônicos (exposição a altas concentrações de compostos químicos prejudiciais ao seu desenvolvimento) ou agudos (biometrias, transporte, extrusão de gametas) foram alvo de poucos estudos (Rodrigues et al., 2007; Cnaani e McLean, 2009; Trusheski et al., 2010; Rodrigues et al., 2015).

Respostas ao estresse

Os peixes são expostos a práticas intrínsecas ao seu manejo rotineiro em aquicultura (biometria, transporte, marcação, extrusão de gametas, vacinação etc.), as quais podem desencadear estresse no animal. O estresse é uma inevitável consequência atribuída a essas práticas, e o seu nível acaba sendo um importante indicador do bem-estar animal (Ruane et al., 1999; Wells e Pankhurst, 1999; Ellis et al., 2004). Após o animal passar pelo período de estresse ao qual foi submetido, sua homeostase é

restabelecida através de um conjunto complexo de respostas adaptativas, a partir de mudanças bioquímicas, fisiológicas e comportamentais (Iwama, 1998; Barton, 2002).

Dependendo da intensidade e da duração do estímulo estressor, o estresse pode ser classificado como agudo ou crônico. No estresse agudo, a resposta adaptativa será resultado de uma exposição por um curto período ao estímulo, enquanto que o estresse crônico é aquele proveniente de uma situação em que o indivíduo esteve sujeito a uma condição desfavorável por um período prolongado (Ortuno et al., 2001).

As respostas adaptativas de estresse da maioria dos peixes seguem um padrão, sendo denominadas respostas primárias, secundárias e terciárias. Muitas respostas primárias e secundárias podem ser acessadas através do sangue ou plasma sanguíneo. Como resposta primária, ocorre ativação do sistema neuroendócrino que resulta na liberação das catecolaminas (adrenalina e noradrenalina) e dos corticosteróides (cortisol). As catecolaminas são originárias de várias partes do corpo, mas principalmente das células cromafins localizadas no rim anterior. Estas células são ativadas sob mediação de fibras colinérgicas pré-ganglionares de nervos simpáticos. As principais funções das catecolaminas estão ligadas ao aumento da freqüência respiratória e cardíaca, aumento da capacidade de transporte de oxigênio e aumento da glicemia. As catecolaminas são liberadas imediatamente após percepção do estímulo estressor, enquanto que a liberação do cortisol ocorre de maneira tardia em relação às catecolaminas (Wendelaar Bonga, 1997; George et al, 2013).

O cortisol é o hormônio corticosteróide mais importante liberado em situação de estresse. É liberado pelas células interrenais também localizadas no rim anterior, estimuladas pelo hormônio adrenocorticotrófico, sob controle do eixo hipotalâmico-pituitário-interrenal. Brânquias, intestino e fígado são tecidos alvos para a ação do

cortisol, pois este atua principalmente na regulação do metabolismo energético e do balanço hidromineral (Barton e Iwama, 1991).

As respostas secundárias são efeitos diretos das primárias, e são principalmente de origem metabólica, hematológica e hidromineral. A hiperglicemia é uma resposta metabólica, mediada por catecolaminas que liberam a glicose do fígado por meio de glicogenólise ou gliconeogênese, atuando como um substrato para a produção de energia. Em situações anaeróbicas, o lactato é liberado como fonte energética para o metabolismo celular (Olsen et al., 1995; Polakof et al., 2012). Como resposta hematológica, peixes submetidos a um estressor tendem a apresentar um maior percentual de hematócrito devido a uma combinação de edema dos eritrócitos e contrações no baço, que libera eritrócitos na corrente sanguínea, aumentando também a concentração de hemoglobinas, numa tentativa de melhorar a capacidade de transporte do oxigênio (Tort et al., 2002; Olsen et al., 2005).

Um dos fatores que afetam as alterações no balanço hidromineral nos peixes é o aumento no consumo de oxigênio (até duas vezes mais em situações de estresse), em que o aumento da concentração de catecolaminas acaba por aumentar a permeabilidade para água e íons nas brânquias, acarretando em um distúrbio iônico, aumentando assim a concentração dos íons sódio, cloreto, potássio e cálcio, em meio hipertônico. O cortisol também possui grande importância para as trocas iônicas com o estímulo da excreção dos íons sódio e cloreto, e em situações crônicas, pode atuar induzindo a apoptose de células branquiais (Barton e Iwama, 1991; Wandelaar Bonga, 1997).

As respostas terciárias estão relacionadas ao organismo como um todo e à população, com a inibição do crescimento, capacidade reprodutiva, resposta imune e

menor capacidade de superar estressores, podendo até mesmo levar o organismo à morte (Barton e Iwama, 1991; Wandelaar Bonga, 1997).

Há poucos estudos sobre o estresse em bijupirá. As alterações dos níveis de cortisol e glicemia em juvenis (300 g) seguiram padrão semelhante ao apresentado por outras espécies após sofrerem a ação de um estressor agudo (emersão por 1 minuto), atingindo picos de cortisol de 62 ng mL^{-1} e de glicemia de 195 mg dL^{-1}, respectivamente, quando mantidos a 24,5°C (Cnaani e McLean, 2009). Trushenski et al. (2010) avaliaram a resposta de estresse de juvenis de bijupirá, expondo-os ao ar ou à redução da coluna d'água, e registraram valores de até 232 ng mL^{-1} para cortisol, 184 mg dL^{-1} para glicose, 439 mOsmol kg^{-1} para osmolalidade e 8,6 mmol L^{-1} para lactato em juvenis de 50 g mantidos a 27,5°C. Os peixes do grupo controle obtiveram valores de 15 ng mL^{-1} para cortisol, 38 mg dL^{-1} para glicose, 368 mOsmol kg^{-1} para osmolalidade e 0,5 mmol L^{-1} para lactato.

Anestésicos

Os peixes são o segundo grupo de animais mais utilizados em experimentos científicos, perdendo apenas para o uso de ratos. Questões legislatórias e éticas que envolvem a utilização e o bem-estar dos peixes demoraram a ser reconhecidas, em comparação aos outros vertebrados (Brown, 2015).

A nocicepção está ligada a um sistema sensorial, via terminações nervosas, que alertam o animal sobre um potencial risco, conectando o cérebro a áreas que permitam ao organismo reagir de forma protetora para evitar um possível dano. Por ser uma resposta imediata, apenas a presença de nociceptores não confirma a teoria de que os

peixes sintam dor e medo (Sneddon et al., 2014). Porém, o seu comportamento frente a um desafio nocivo, demonstrando capacidade de memória em longo prazo e percepção para antecipação, nos permite inferir que de fato os peixes são capazes de experimentar sensações homólogas à dor e ao medo (Sneddon, 2003; Braithwaite e Boulcott, 2007; Sneddon et al., 2014).

Rose et al. (2014) confrontaram este fato, afirmando que peixes não possuem a capacidade de sentir dor como os humanos por não ter cognição complexa, e que sua resposta nociceptiva é limitada. No entanto, vários trabalhos (Sneddon, 2003; Chandroo et al., 2005; Braithwaite e Boulcott, 2007) provam esta capacidade em peixes, e Brown (2015) criticou o trabalho de Rose et al. (2002) por ser antropomórfico e antropocêntrico, não levando em consideração os múltiplos níveis de consciência. De acordo com Sneddon et al. (2014), mais importante do que provar a capacidade de um animal sentir medo, é tratá-lo com respeito e garantir seu bem-estar. Assim, a utilização de anestésicos se torna importante para evitar possíveis injúrias e estresse durante a manipulação dos peixes, sendo imprescindível o estudo sobre diferentes anestésicos para diversas espécies, bem como suas implicações fisiológicas (Guénette et al., 2007).

Para isso, primeiramente, é importante diferenciar sedação de anestesia. Sedação seria um estágio anterior a anestesia, onde em sedação profunda, há baixa percepção sensorial e redução da respiração, porém o peixe ainda não perdeu totalmente o equilíbrio e ainda responde a estímulos grosseiros. Em anestesia profunda, há a perda total do equilíbrio e do tônus muscular e o batimento opercular (ventilação) é quase imperceptível (Ross e Ross, 2008).

É difícil lidar com um peixe não anestesiado no manejo, e lesões epiteliais podem ocorrer durante a tentativa de contenção. Assim, anestésicos são bastante

utilizados em práticas de manejo em aquicultura, como biometrias, coletas de sangue, cirurgia, marcação entre outras atividades (Trushenski et al., 2013). De acordo com Marking e Meyer (1985), um anestésico ideal deve ter como características: não apresentar toxicidade ao peixe ou a quem o maneje; o peixe deve ser anestesiado em até 3 minutos e ter sua recuperação completa em até 5 minutos; não deixar resíduos após período de depuração; além de ter custo acessível.

A eficácia de um anestésico varia principalmente de acordo com fatores biológicos e ambientais. Dentre os fatores biológicos, a eficácia pode ser diferente entre espécies, tamanho/ peso, maturidade sexual, nível de estresse, condição corporal e saúde do animal. Temperatura, pH, salinidade e concentração de minerais são fatores ambientais que interferem na atuação do anestésico (Ross e Ross, 2008).

Os anestésicos mais utilizados mundialmente são a tricaína metanosulfato (MS-222), eugenol, benzocaína, 2-fenoxietanol e metomidato (Husen e Sharma, 2014). O principal modo de ação do MS-222 ($H_2NC_6H_4CO_2C_2H_5 \cdot CH_3SO_3H$) é suprimindo o sistema nervoso, onde a excitabilidade do nervo é restringida pelo bloqueio dos canais de sódio. No corpo, o anestésico é metabolizado por acetilação e excretado pelas brânquias e rim (Burka et al., 1997; Carter et al., 2011). O MS-222 é o único anestésico aprovado, pelo órgão dos Estados Unidos que controla a indústria alimentícia e farmacêutica (Food and Drugs Administration – FDA) e por órgão análogo no Canadá (Health Canada), para ser usado em peixes destinados ao consumo humano. Porém os peixes só podem ser consumidos após um período de 21 dias de depuração (Meinertz e Schreier 2009; Trushenski et al., 2013).

O eugenol está no topo da lista dos anestésicos candidatos à aprovação perante a FDA, já com patrocinadores contratados e dispostos a realizar este feito. Apesar de

ainda não ser aprovado, já é considerado como um novo fármaco em fase de investigação para animais (Investigational New Animal Drug – INAD) (Trushenski et al., 2013). Para uso em humanos, ele é liberado pela FDA e amplamente utilizado em medicina e odontologia (Cho e Heath, 2000). O anestésico eugenol (4-alil-2-metoxifenol) é o princípio ativo do óleo de cravo, extraído das folhas, caules e flores de árvores da espécie *Syzygium aromaticum* (Javahery et al., 2012). Ele atua bloqueando receptores vaniloides que estão ligados à transmissão da dor. Ele ainda interage com neurotransmissores envolvidos na percepção de dor, possuindo efeito agonista ao GABA (ácido gama-aminobutírico) e antagonista ao NMDA (N-metil D-aspartato). GABA é um neurotransmissor inibitório do sistema nervoso central. Já o NMDA é um receptor ativado pelo glutamato, maior neurotransmissor excitatório do cérebro. A combinação do efeito positivo na atividade dos receptores GABA e do efeito negativo para os receptores NMDA pode ser a principal responsável pelo efeito anestésico do eugenol (Yang et al., 2003; Guénette et al., 2007; Rao e Finkbeiner, 2007; Watt et al., 2008).

Assim como o eugenol, o mentol (2-isopropyl-5-methyl-cyclohexanol) também é um anestésico adquirido a partir de extrato vegetal. Ele é obtido de plantas do gênero *Mentha*, e é um importante produto para indústria farmacêutica e alimentícia (Eccles, 1994; Hoshiba et al., 2015). Também não é aprovado para uso em peixes, porém é facilmente encontrado no mercado e a baixo preço (Façanha e Gomes, 2005). Atua de duas principais maneiras: modulando positivamente o receptor GABA e bloqueando os canais de sódio voltagem dependentes (Watt et al., 2008; Kasai et al., 2014).

Gullian e Villanueva (2009) utilizaram os anestésicos MS-222 e eugenol para anestesiar juvenis de bijupirá com peso de 5 a 14 g, e as concentrações ideais

encontradas foram 60 mg MS-222 L^{-1} e 20 mg eugenol L^{-1}, em uma temperatura de 23ºC. Trushenski et al. (2012) utilizaram vários métodos (150 mg MS-222 L^{-1}; 60 mg eugenol L^{-1}; 150 mg benzocaína L^{-1}; 750 mg CO_2 L^{-1}; eletrossedação pulsada: 100 V, 30 Hz, ciclo de 25% por 5 segundos) para anestesiar bijupirás de 300 g a 27°C, e todos foram eficientes.

Transporte

O transporte é uma prática inerente a aquicultura, sendo utilizado para diversos fins, como por exemplo, levar os peixes do berçário aos locais de engorda, que muitas vezes se encontram distantes. Além disso, outros exemplos são: transporte de peixes selvagens para laboratórios, para comercialização (alimento/ ornamental), para práticas recreativas, para aquários e mesmo para pesquisa (Lim et al., 2003; Iversen et al., 2009; Sampaio e Freire, 2016).

Há dois tipos de sistema de transporte: o transporte aberto e o fechado. Uma das principais características do sistema aberto é o contínuo fornecimento de oxigênio e/ou ar pressurizado, permitindo a retirada do excesso de CO_2. Já o sistema fechado utiliza unidades de transporte lacradas com injeção de oxigênio, como sacos de polietileno, por exemplo (Berka, 1986). O transporte fechado é amplamente empregado por todo o mundo, e no Brasil é o principal sistema utilizado, sendo considerado mais econômico (Berka, 1986; Golombieski et al., 2003). Porém possui limitações, sendo a mais importante delas a deterioração da qualidade da água (Amend et al., 1982; Berka, 1986). Uma sequência de fatores leva a essa degradação: os peixes já estão estressados pelo manejo pré-transporte e agora se encontram em um ambiente confinado; aumentam sua

atividade respiratória, o que diminui ainda mais a concentração limitada de oxigênio; o consumo de oxigênio leva a um aumento do dióxido de carbono, acidificando a água, o que reduz o pH; a amônia excretada pode se tornar letalmente tóxica (Paterson et al., 2003).

As mudanças que ocorrem na qualidade da água afetam as respostas fisiológicas de estresse nos peixes. Além das altas concentrações de CO_2 acidificarem a água, também acidificam o plasma sanguíneo. Tanto o CO_2 como os íons H^+ podem se ligar à hemoglobina, afetando sua afinidade com o oxigênio. O aumento na concentração de íons H^+, reduzindo o pH e diminuindo afinidade da hemoglobina pelo oxigênio é caracterizado como efeito Bohr. Além do efeito Bohr, há também o efeito Root, onde a capacidade de transporte de oxigênio do sangue é drasticamente reduzida em sangue com baixo pH, sendo o efeito necessário principalmente para preencher a bexiga natatória e fornecer oxigênio para a retina, que não possui capilares (Souza e Bolina-Rodriguez, 2007). O aumento do hematócrito e da concentração de hemoglobinas são formas do organismo superar esta condição e aumentar o oxigênio sanguíneo (Paterson et al., 2003; Rummer e Brauner, 2011).

Outra forma de superar o aumento do CO_2 e a redução do pH sanguíneo, é através do balanço ácido-base. As reações catalisadas pela enzima anidrase carbônica são representadas pela fórmula: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. Assim, o organismo é favorecido para que o dióxido de carbono (CO_2) e o bicarbonato (HCO_3^-) estejam sempre em equilíbrio (Claiborne e Heisler, 1986). O aumento do bicarbonato para a manutenção do pH está ligado principalmente aos processos de troca entre os íons Na^+/ H^+ e Cl^-/ HCO_3^- no eritrócito, sendo que o H^+ é excretado e o Na^+ absorvido, enquanto que o HCO_3^- é excretado e o Cl^- absorvido (Perry e Gilmour, 2006). Este balanço ácido-

base tem grande importância ionorregulatória: para peixes de água doce, ajudará a manter a osmolalidade plasmática; já peixes marinhos correm o risco de aumentar a carga de Na^+ e Cl^- que eles normalmente já enfrentam, alterando seu padrão osmorregulatório (Claiborne et al., 2002).

Na tentativa de diminuir o estresse dos peixes no transporte e garantir sua sobrevivência durante e depois do procedimento, algumas medidas podem ser tomadas, tais como: determinar densidade de estocagem ideal; injeção de oxigênio puro nos sacos de transporte; utilização de tampões para a manutenção do pH; salinidade próxima ao ponto isosmótico; redução da temperatura e utilização de anestésicos (Lim et al., 2003; Sampaio e Freire, 2016).

Densidade de estocagem

A quantidade de peixes a ser transportada por unidade de área ou volume (densidade de transporte) vai depender principalmente da qualidade da água, temperatura, tamanho do peixe e espécie e tempo de transporte (Ashley, 2007). Em regra, quanto maior o período do transporte, menor deve ser a densidade de estocagem (Berka, 1986; Harmon, 2009). Entretanto, quanto maior a densidade, menor o custo do transporte, porém altas densidades podem prejudicar a saúde do animal, e até mesmo aumentar as taxas de mortalidade. Por isso densidades apropriadas devem ser avaliadas para um transporte seguro e economicamente viável (Sampaio e Freire, 2016).

Juvenis de matrinxã *Brycon amazonicus* (13 g) foram transportados por 4 h (25 – 27°C) em três diferentes densidades (86, 125 e 166 g L^{-1}) e não houve mortalidades (Urbinati et al., 2004). Já juvenis de tambaqui *Colossoma macropomum* (52 g) transportados por 10 h (28°C) nas densidades 78, 156, 234 e 312 g L^{-1}, apresentaram

mortalidade acumulada (96 h após transporte) de 32, 43 e 65% nas três maiores densidades, sendo a densidade de 78 g L^{-1} considerada a ideal para o transporte da espécie (Gomes et al., 2003). É notável a necessidade de verificação da densidade de estocagem ideal para cada espécie em suas diferentes fases de vida e diferentes condições de transporte como temperatura, pH e salinidade

Colburn et al. (2008) transportaram juvenis de bijupirá com peso de 1,5 – 3 g por 24 h, em diferentes densidades (5 – 25 g L^{-1}) e diferentes temperaturas (19 – 25°C), onde ficou evidenciado que houve baixa mortalidade em densidades de até 20 g L^{-1}. Liao et al. (2004) observaram que um dos maiores problemas na produção de bijupirá está relacionada a alta mortalidade no transporte dos animais do berçário às gaiolas de engorda, e neste mesmo estudo os autores afirmam que os juvenis devem atingir pelo menos 30 g para serem transportados.

Tampões de pH

Os tampões podem ser adicionados durante o transporte para manter o pH da água estável, já que nesses procedimentos ocorre a sua acidificação devido ao aumento do CO_2. Os mais utilizados são bicarbonato de sódio, TRIS (hidroximetil metilamina) e magnosferas (Treasurer, 2012).

O uso de bicarbonato de sódio (1 e 2 g L^{-1}) foi eficiente para manter os níveis de pH durante o transporte (24 h/ 15°C) de *Gadus morhua* (12 g L^{-1}).

Salinidade

Pelo fato de a salinidade ser uma variável controlável e a osmorregulação ser um processo que despende energia, o crescimento dos peixes pode ser maximizado pela

seleção de salinidades que diminuam os gastos energéticos com as trocas iônicas (Sampaio e Bianchini, 2002). Normalmente, os peixes possuem menor gasto energético com osmorregulação quando estão em ambientes com salinidade próximas ou equivalentes ao ponto isosmótico do plasma (Gaumet et al., 1995; Imsland et al., 2001). O ponto isosmótico do bijupirá se encontra na salinidade de 11,2 g L^{-1} (Burkey et al., 2007).

O bijupirá é considerado como sendo uma espécie oceânica, porém quando juvenis, são comumente encontrados em áreas próximas à costa e baías (Shaffer e Nakamura, 1989). Por ser uma espécie eurialina, Resley et al. (2006) realizaram estudos sobre crescimento e sobrevivência de juvenis de bijupirá (7 g) nas salinidades 5, 15 e 30 g L^{-1}. A sobrevivência na salinidade 5 g L^{-1} foi significativamente menor (68,9%) que nas salinidades 15 (90%) e 30 (92,5%) g L^{-1}.

Em transporte de peixes de água doce, é comum a adição de cloreto de sódio para aumentar a salinidade e diminuir o estresse devido ao menor gasto de energia despendido para a osmorregulação. Para o transporte de espécies marinhas, estudos também sugerem a diluição da água salgada para que fique próxima ao ponto ismosmótico da espécie, com a mesma finalidade de diminuir o gasto energético (Harmon, 2009; Sampaio e Freire, 2016).

Stieglitz et al. (2012) transportaram alevinos de bijupirá (1,65 g) por 24 h (19°C) em diferentes densidades (5, 10, 15 e 20 g L^{-1}) e diferentes salinidades (12 e 32 g L^{-1}). Ficou evidenciado por esses autores que não houve mortalidade para a densidade 5 g L^{-1} em ambas as salinidades. Para a densidade 15 g L^{-1}, a sobrevivência foi de 30% na salinidade 32 g L^{-1}, enquanto que na salinidade 12 g L^{-1} foi de 80%. Para a densidade de 20 g L^{-1}, a sobrevivência foi de 10% para a maior salinidade e de 65% para a menor.

Temperatura

A temperatura afeta todos os aspectos fisiológicos dos peixes por ter influência nos processos que envolvem propriedades fisiológicas de moléculas e macromoléculas biológicas. O aumento na temperatura tem como consequência diversos fatores, como o aumento da toxicidade dos contaminantes dissolvidos, o desenvolvimento de patógenos, a diminuição da concentração de oxigênio dissolvido, aumento na temperatura corporal, assim como na taxa metabólica dos animais. Assim, a saúde dos peixes em meios intensivos de criação é, ou pode ser afetada pelas variações e extremos de temperatura. Já o resfriamento da água diminui a temperatura do corpo, desacelera a resposta imune, além de reduzir a alimentação, atividade e crescimento (Skjervold et al., 2001; Crockett e Londraville, 2006; Falcon et al., 2007).

Para manter a qualidade da água no transporte, a redução da temperatura da água é uma técnica bastante utilizada com o objetivo de reduzir o metabolismo dos peixes, que pode ser até triplicado nessas condições. Porém é preciso ter cuidado durante o resfriamento, pois quedas muito bruscas podem ser igualmente estressantes aos peixes (Lim et al., 2003; Harmon, 2009).

Golombieski et al. (2003) transportaram alevinos (1 – 2,5 g) de jundiá *Rhamdia quelen* em sistema fechado testando quatro densidades (50, 67, 87 e 168 g L^{-1}), três temperaturas (15, 20 e 25°C) e três períodos de transporte (6, 12 e 24 h). Houve mortalidade apenas na maior densidade após 24 h de transporte para as temperaturas 20 e 25°C. Os resultados mais notáveis estão para as alterações do oxigênio dissolvido e do dióxido de carbono na maior densidade. Enquanto que para 15°C foi observado 18 mg O_2 L^{-1}, para 20 e 25°C observou-se 0,60 e 0,44 mg O_2 L^{-1}, respectivamente. Já para

CO_2, observou-se uma concentração de 61 mg CO_2 L^{-1} em 15°C, e 98 e 126 mg CO_2 L^{-1} para 20 e 25°C, respectivamente.

Anestésicos

O uso de anestésicos durante o transporte tem sido bastante empregado, com a finalidade de reduzir o metabolismo dos peixes e assim reduzir as respostas de estresse provenientes desta condição. Com a diminuição da taxa metabólica, os peixes consomem menos oxigênio, liberam menos CO_2, excretam menos amônia e assim há uma menor deterioração da qualidade da água (Iversen et al., 2009; Sampaio e Freire, 2016).

Apesar de o anestésico ter a função de minimizar as respostas fisiológicas, muitas vezes ele mesmo pode causar o estresse (Velisek et al., 2011). Na Tabela 1 podemos ver a eficiência de alguns anestésicos utilizados em transporte e suas concentrações para diferentes espécies.

Azambuja et al. (2011) transportaram jundiá (64 g) por diferentes períodos (5, 6 e 7 h – 22 °C) com o óleo essencial de *Lippia alba* (10 mg L^{-1}), e constataram que a adição do anestésico foi eficiente em melhorar o estado oxidativo nos tecidos.

Juvenis de linguado *Paralichthys orbignyanus* (13 g) transportados por 7 h (22,5 °C) com óleos essenciais de *Aloysia gratissima* e *Ocimum gratissimum* apresentaram diferentes respostas em relação aos anestésicos. Quando transportados com 90 e 130 mg L^{-1} de *A. gratissima*, ocorreram 8 e 100% de mortalidade, respectivamente. Já para *O. gratissimum*, a concentração de 10 mg L^{-1} se mostrou eficiente em reduzir parâmetros de estresse (Benovit et al., 2012).

Para um transporte de juvenis de bijupirá (30 g) por 8 h (22,5 °C) em uma densidade de 10 g L^{-1} com diferentes concentrações de benzocaína (0, 2 e 6 mg L^{-1}), não houve mortalidade. Porém, após o transporte, os peixes apresentaram o dobro dos níveis de glicemia no tratamento com 6 mg benzocaína L^{-1} quando comparado ao tratamento sem o anestésico. Além disso, apenas os peixes transportados com 2 mg benzocaína L^{-1} não recuperaram seus níveis de glicemia após 48 h do transporte. Assim, os autores não recomendam o uso de benzocaína no transporte de bijupirá (30 g) (Pedron et al., 2016).

Tabela 1: Eficiência de concentrações de anestésicos utilizados em transporte de peixes.

Espécie	Anestésico	Eficácia	Autores
Brycon amazonicus	Benzocaína	Não	Carneiro et al., 2002
Centropomus paralelus	Mentol	Não	Sepulchro et al., 2016
Coreius guichenoti	MS-222	30 mg L^{-1}	Zhao et al., 2014
Micropterus salmoides	Óleo de cravo	5-9 mg L^{-1}	Cooke et al., 2004
Oreochromis niloticus	Mentol, Benzocaína e Eugenol	M: 75 mg L^{-1} B: 20 mg L^{-1} E: 20 mg L^{-1}	Navarro et al., 2016
Oreochromis niloticus	Óleo essencial de *Lippia alba*	20 mg L^{-1}	Hohlenwerger et al., 2017
Paralichthys orbignyanus	Óleos essenciais de *Aloysia gratissima* e *Ocimum gratissimum*	Ag: Não Og: 10 mg L^{-1}	Benovit et al., 2012
Pleuronectes americanus	Lidocaína	5, 10 e 20 mg L^{-1}	Park et al., 2009
Rachycentron canadum	Benzocaína	Não	Pedron et al., 2016
Rhamdia quelen	Óleo essencial de *Lippia alba*	10 mg L^{-1}	Azambuja et al., 2011
Rhamdia quelen	Eugenol e extrato de *Condalia buxifolia*	E: 2,5 mg L^{-1} Cb: 50 mg L^{-1}	Becker et al., 2013
Rhamdia quelen	Óleo essencial de *Aloysia triphylla*	30-50 mg L^{-1}	Parodi et al., 2014
Salmo salar L.	Metomidato	1 mg L^{-1}	Sandodden et al., 2001
Salmo salar L.	Aqui-S	5 mg L^{-1}	Iversen e Eliassen, 2009

Assim, torna-se necessário estudar e compreender as respostas fisiológicas de estresse do bijupirá em práticas comuns em aquicultura, como a utilização de anestésicos e técnicas de transporte, visando sua saúde e bem-estar.

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HIPÓTESES

- O transporte é um fator estressor para juvenis de bijupirá. O uso de anestésicos e o manejo de temperatura e salinidade podem minimizar as respostas de estresse.
- Os anestésicos, apesar de permitirem o manuseio dos peixes, podem ser um agente estressor para juvenis de bijupirá.

OBJETIVOS

Objetivo geral

Estabelecer concentrações ideais de anestésicos e melhores condições de transporte para juvenis de bijupirá *R. canadum.*

Ojetivos específicos

- Determinar a densidade de estocagem ideal para o transporte de bijupirá.
- Verificar a temperatura e a salinidade adequada para o transporte de juvenis de bijupirá.
- Determinar a concentração ideal do anestésico mentol, bem como sua utilização no transporte de juvenis de bijupirá.
- Determinar a concentração ideal dos anestésicos MS-222 e eugenol para juvenis de bijupirá.

Capítulo l

Transport of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) at different stocking densities

Artigo submetido à revista Aquaculture Research.

Abstract

This experiment aimed to determine the appropriate stocking density for transport of juvenile cobia studying their survival and blood stress response. Fish (29.4 g) were placed in 60 L polyethylene bags (10 L of seawater, 20 L of oxygen and 1 g $NaHCO_3$ L^{-1}) at three different densities (15, 24, and 33 g L^{-1}). Then they were transported by truck (8 h) in styrofoam boxes (21.5ºC). Before transport, nine fish were sampled for blood collection as Control group. Blood samples were also taken right after transport (0 h), 2 and 24 h after for determination of glucose, osmolality, hematocrit, hemoglobin, lactate, pO_2, pH, pCO_2 and HCO_3^-. Mortality was 18.2% for cobia transported at the highest density, but no mortalities were observed up to 24 g L^{-1}. Water quality was compromised at the highest stocking densities, hypercapnia and hypoxia were observed at 24 and 33 g L^{-1}, while pH was lower (7.08) at the highest density. Regarding blood composition, hyperglycemia was observed for densities 24 and 33 g L^{-1} at 0 and 2 h after transport. At the highest density, hyperlactatemia and low hemoglobin concentration were observed 2 h after transport. Even with an increase in blood pCO_2 levels, cobia were able to cope and keep pH, releasing HCO_3^-. According to the results shown in this study, it is recommended to buffer the water used to transport juvenile cobia (30 g) in a closed system (1 g $NaHCO_3$ L^{-1}), thus increasing the safe stocking density to 24 g L^{-1}.

Running title: Transport of cobia using a pH buffer.

Keywords: Rachycentridae, fish transport, sodium bicarbonate, blood parameters.

Introduction

Fish are subjected to many stressful procedures in aquaculture, including live transport. Transport is considered a multiphase operation and the optimization of a stocking density for a determined period is crucial to reduce stress response and mortality, during and after transport (Pakhira, Nagesh, Abraham, Dash & Behera 2015). The fish stress can be accessed by biochemical and hematological analyses (Svobodová, Máchová, Drastichová, Groch, Lusková, Poleszczuk, Velisek & Kroupová 1999).

The maintenance of good water quality during transport of live fish in closed system is essential to reduce stress related responses, as water quality deprivation could compromise fish health and welfare (Harmon 2009; Stuart, Losordo, Olin & Drawbridge 2015). The main problems during transport are the build up of ammonia and carbon dioxide and the decrease in oxygen and pH levels (Amend, Croy, Goven, Johnson & McCarthy 1982; Treasurer 2010). Buffers can be added to the water in order to keep appropriate pH levels, among them sodium bicarbonate, tris (hydroxymethyl) methylamine, and magnaspheres (Treasurer 2012). Sodium bicarbonate was effective to maintain pH levels during transport of juvenile cod *Gadus morhua* L. (Treasurer 2010) and it is easily found in the market at a relatively low cost.

Liao, Huang, Tsai, Hsueh, Chang & Leano (2004) recommended that cobia *Rachycentron canadum* (Linnaeus) should weigh at least 30 g prior to be stocked in cages, the main production system for this species around the world. Pedron, Miron, Rodrigues, Okamoto, Tesser & Sampaio (2016) evaluated the role of an anesthetic, benzocaine, on the transport of ≈ 30 g cobia in a closed system, stocking density was 10 g L^{-1}, and no buffers were added. Despite a reduction in pH, no mortalities were

observed after 8 h. Cobia (30 g) transported at three different densities (10, 20, and 30 g L^{-1}) for 8 h without buffering showed mortality (≈ 40 %) at the highest density (Pedron 2013). Therefore, the aim of this study was to evaluate the effects of different stocking densities on the stress responses of juvenile cobia (≈ 30 g) transported in plastic bags.

Materials and Methods

Animals

Fish were acquired from a commercial hatchery and raised at 26.5°C and salinity 30 g L^{-1} in a recirculating aquaculture system (RAS) at a density of 3 g L^{-1}. Feed was supplied for juvenile cobia twice daily with a commercial diet (57% crude protein; 14.5% lipid - NRD, INVE, Grantsville, UT, USA). Experiments were approved by Ethical and Animal Welfare Committee of the Federal University of Rio Grande – FURG (Certificate number 020/2014).

Transport

Fish (29.4±5.6 g, 18.3±1.2 cm) were fasted 24 h before transport. Temperature in the RAS was reduced to 21.5ºC within the last 3 h (1.6°C per hour), before fish were packed. Three stocking densities were tested: 15, 24, and 33 g L^{-1}, all in triplicate. A total of 216 fish were placed in 60 L polyethylene bags filled with 10 L of salt water (30 g L^{-1}), 20 L of oxygen and 1 g L^{-1} of pH buffer sodium bicarbonate $NaHCO_3$. Beyond that, three extra bags without fish were packed for water quality analysis. The bags were packed in styrofoam boxes at 21.5ºC and then transported by truck for 8 h.

At the end of transport, temperature and DO were measured with YSI Model 550A meter (Yellow Springs Instruments, Yellow Springs, Ohio, USA), and the pH was measured with the pHmeter (FE20-FiveEasyTM, Mettler Toledo, Schwerzenbach, Switzerland). Alkalinity was measured following APHA (1999) and CO_2 was calculated with the software CO_2 Analysis Salt® (Timmons & Ebeling 2010). TAN was determined accordingly to Solorzano (1969) and NH_3-N was calculated using the equations of Ostrensky, Marchiori & Poersch (1992) adapted from Whitfield (1974).

Sampling

Before transport, nine fish were sampled for blood collection as a Control group. Blood of three fish was sampled from three bags per treatment (triplicate) immediately on arrival (0 h), after transport, and three fish from each of the remaining bags were placed in recovering tanks (one tank for each bag) filled with 50 L of salt water. Fish were sampled 2 and 24 h later. Benzocaine at 50 mg L^{-1} was used during the samples to ease handling. Blood was collected from the caudal vein with a heparinized 1 mL syringe. An I-STAT Portable Clinical Analyzer (Abbott Laboratories, Chicago, Illinois, USA) coupled to an $CG8^+$ cartridge was used to measure blood properties: glucose, hematocrit, hemoglobin, pH, partial gas pressure of CO_2 (pCO_2) and O_2 (pO_2), displaying calculated values of blood bicarbonate (HCO_3^-) and O_2 (pO_2). Values for pCO_2 and HCO_3^- were corrected to the experimental temperature according to the manufacture´s specifications. The efficacy of I-STAT measurements has been proved for several fish species, like bonefish *Albula vulpes* L. (Cooke, Suski, Danylchuk, Danylchuk, Donaldson, Pullen, Bulte, O'toole, Murchie, Koppelman, Shultz, Brooks & Goldberg 2008), Atlantic salmon *Salmo salar* L. (Kristensen, Rosseland, Kiessling,

Djordevic & Massabau 2010), Atlantic halibut *Hipoglossus hipoglossus* L. (Paust, Foss & Imsland 2011), tambaqui *Colossoma macropomum* (Cuvier) (Barbas, Stringheta, Garcia, Figueirerdo & Sampaio 2016), including cobia (Rodrigues, Pedron, Romano, Tesser & Sampaio 2015). Blood was centrifuged (10,192 x *g*) for 10 min. (4°C) for plasma osmolality measurements using a vapor pressure osmometer (Vapro 5520; Wescor, Inc.; Logan, Utah, USA) and lactate was determined using a portable meter (Accutrend Plus®, Roche, Mannheim, Germany).

Statistical analyses

Water quality and blood parameters were analyzed by one-way ANOVA, followed by Tukey test when significant differences were observed. Dunnett test was applied to identify data points that were significantly different from Control. All analyzes were performed with a minimal significant level of $p<0.05$. All values were presented as mean and standard deviation.

Results

Cobia survived transport in a closed system for 8 h at densities up to 24 g L^{-1}. However, with stocking density of 33 g L^{-1}, mortality reached 18.2% right after transport.

Dissolved oxygen was significantly lower ($p<0.05$) when cobia were transported at 24 and 33 g L^{-1}, it decreased 4-fold at the highest stocking density, compared to bags transported without fish. However, the concentration of dissolved oxygen was above 10 mg O_2 L^{-1} at the lowest stocking density. Carbon dioxide increased up to 56 mg CO_2 L^{-1}

as the stocking density was increased. Rich CO_2 environment favored reduction of pH levels, which was lower for the highest stocking density. Alkalinity was significantly different ($p<0.05$) among stocking densities, however it was always above 760 mg $CaCO_3$ L^{-1}. Total (TAN) and unionized ammonia levels were significantly higher at all densities compared to treatment Without Fish ($p<0.05$). However, levels of TAN and unionized ammonia were all below 0.30 $NH_4^+ + NH_3$-N mg L^{-1} and 0.01 mg NH_3-N L^{-1}, respectively (Table 1).

Table 1.

The values obtained for blood parameters before and after transport are shown in tables 2 and 3. Within 0-2 h after transport glucose was significantly higher for cobia transported at the densities 24 and 33 g L^{-1} compared to the Control and to cobia transported at the lowest stocking density. However, after 24 h, glucose levels in all treatments had returned to the Control level.

There were no significant differences for osmolality at 15 and 24 g L^{-1} when compared with Control, independent of time. On the other hand, despite a significant rise in osmolality during the first 2 h after transport, it returned to Control levels within 24 h for cobia transported at 33 g L^{-1}. Hematocrit levels were all similar to Control, and there were no significant differences at each stocking density along 24 h ($p>0.05$). Hemoglobin concentration was not affected at the lowest and highest stocking density. However, hemoglobin was higher than Control immediately after transport when stocking density was 24 g L^{-1}. Within the same treatments, there were no significant differences along time. Lactate concentration was higher than Control just at 2 h for 33 g L^{-1}. Right after transport (0 h), lactate levels were higher at 33 g L^{-1} when compared to the other densities. There were no significant differences for pO_2 concentrations.

Table 2.

Table 3.

Blood pH rose significantly on arrival ($p<0.05$), but after 2 h it was above the Control level only for cobia transported at the highest stocking density. Nevertheless, after 24 h pH levels were all similar among stocking densities and also to the Control ($p<0.05$). There was an increase in blood pCO_2 for all densities right after transport ($p<0.05$), the higher the stocking density, the higher was the pCO_2. However, as soon as 2 h after transport, pCO_2 decreased at the lowest stocking density, and it remained lower than the Control after 24 h, although at the time, there was no difference among pCO_2 and stocking densities at 2 and 24 h. HCO_3^- levels also increased after transport, however after 2 h all treatments were already similar to Control.

Discussion

The increase in stocking density during fish transportation could be associated with the deterioration in water quality (Golombieski, Silva, Baldisserotto & Silva 2003). Thus, the techniques used in aquaculture during live transport are mainly related to reduction of the stress responses and to improve water quality. The main problems in closed system transport are the buildup of ammonia and CO_2, coupled to decrease of oxygen and pH. The pH decrease could be minimized by the addition of pH buffers, like sodium bicarbonate (Ashley 2007; Harmon 2009).

The decline in water pH through live transport was also reported for other species (Carneiro, Kaiseler, Swarofsky & Baldisserotto 2009; Pakhira et al. 2015). Treasurer (2012) recommended 2 mg $NaHCO_3$ L^{-1} to transport juvenile cod *G. morhua*

L. in open systems. In the present work, pH decreased with the increase in stocking densities, but all remained above 7.1, a level considered safe, as pH above 6.5 is not harmful for cobia (Rodrigues et al. 2015).

The lethal concentration for TAN and unionized ammonia for cobia are 38.5 and 1.13 mg L^{-1}, respectively (Rodrigues, Schwarz, Delbos & Sampaio 2007). However, in this study the highest TAN and unionized ammonia were only 0.27 mg TAN L^{-1} at density 33 g L^{-1} and 0.0025 N-NH_3 L^{-1} at 15 mg L^{-1}, so these parameters were not an issue for cobia.

It is known that oxygen consumption is related with CO_2 excretion, and that CO_2 concentrations above 40 mg L^{-1} are considered harmful for oxygen transport through tissues (Wedemeyer 1996; King 2009). In this study, the lowest dissolved oxygen concentration in the water was 3.29 mg L^{-1} at the highest density. Concomitantly, at 33 g L^{-1} there were the highest CO_2 levels (56 mg L^{-1}) and it was the only treatment that presented mortality (18.2%). Indeed, hypoxia and hypercapnia are related to mortality during fish transport (Tang, Thorarensen, Brauner, Wood & Farrell 2009).

Stress response is mediated by catecholamines and corticosteroids, leading to hyperglycemia, hyperlactatemia, osmolality disturbance, increase in oxygen uptake and changes on hemoglobin oxygen affinity (Carragher & Rees 1994; Wendelaar Bonga 1997). In the present study, glucose levels were higher at the end of transport and remained high after 2 h at 24 and 33 g L^{-1}, reaching 375 mg dL^{-1} at the highest density. Trushenski, Schwarz, Takeuchi, Delbos & Sampaio (2010) submitted cobia to different stress conditions, and the highest glucose was 189 mg dL^{-1} after 1 h from an air exposure challenge (1 min.). Therefore, cobia faced an intense stress condition when transported at 33 g L^{-1}, thus culminating in their mortality.

For marine fish, which lives in hyperosmotic environment, it is common to observe an increase in osmolality levels in stressfull situations due to the increase in gills permeability to water and ions (Wendelaar Bonga 1997). Despite a higher osmolality at 0 and 2 h for 33 g L^{-1}, after 24 h it returned to baseline levels. This seems to be a typical response of cobia following an acute exposure to a stressor. Cobia challenged with air exposure (1 min.) also presented higher osmolality levels (439 mOsm kg^{-1}) after 0.5 h from exposure, nonetheless they returned to baseline levels after 1 h from challenge (Trushenski et al. 2010).

When fish face a situation that demands more energy to cope with higher metabolic rates, catecholamines are released and raise hematocrit and hemoglobin concentrations to increase oxygen carrying capacity (Wendelaar Bonga 1997; Dobšíková, Svobodová, Blahová, Modrá & Velíšek 2006). In this study, hemoglobin concentration increased on arrival only for cobia transported at 24 g L^{-1}. On the other hand, there was no difference for hematocrit related to Control, and the same pattern was found for cobia transported with benzocaine (Pedron et al. 2016). Despite no significant difference, hemoglobin concentration decreased at the highest density after 2 h (5 mmol L^{-1}), concomitantly to a considerably and significant increase in lactate. When the oxygen supply is not enough for aerobic metabolism and thus fish feature an anaerobic condition, lactate levels increase (Olsen, Einarsdottir & Nilssen 1995). Blood oxygen concentrations were not different among treatments or Control, however it is remarkable that hemoglobin concentration was lower at the highest density 2 h post-stress, therefore a lower oxygen carrying capacity could have led to higher lactate levels. Rainbow trout *Oncorryhnchus mykiss* (Walbaum) transported in an open system for 3 h with a density of 169 g L^{-1} also showed an increase in lactate levels after

transport, returning to baseline levels after 24 h (Shabani, Erikson, Beli & Rexhepi 2016).

When blood CO_2 is elevated, the organism copes with acidity by increasing HCO_3^- concentration, thus sustaining pH levels (Claiborne & Heisler 1986). This was exactly what happened for cobia after transport: the CO_2 concentration increased along with pH and HCO_3^- levels. Silver catfish *Rhamdia quelen* transported for 6 h in plastic bags with different concentrations of essential oil of *Lippia alba* also presented an increased in blood CO_2 and HCO_3^- levels after transport (Becker, Parodi, Zeppenfeld, Salbego, Cunha, Heldwein, Loro, Heinzmann & Baldisserotto 2016).

There was 18.2% of mortality at the highest density (33 g L^{-1}) in this study. Cobia (30 g) transported for 8 h without buffering also showed mortality (≈ 40%) at the highest density (30 g L^{-1}), when pH reached 6.4 (Pedron 2013). Therefore, the maximum safe stocking density to transport juvenile cobia is 24 g L^{-1}, as long as pH does not fall below 7.2.

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Tables

Table 1: Water quality parameters (mean ± SD) measured immediately after 8 h transport of juvenile cobia *Rachycentron canadum* at different stocking densities (15, 24 and 33 g L^{-1}). Different letters indicate significant differences ($p<0.05$) among treatments, as indicated by one-way ANOVA and Tukey's test.

Parameters	**Densities (g L^{-1})**			
	Without Fish	**15 g L^{-1}**	**24 g L^{-1}**	**33 g L^{-1}**
DO	18.75±1.20^{a}	10.23±3.11^{b}	4.26±1.26^{c}	3.29±0.87^{c}
pH	7.82±0.02^{a}	7.47±0.05^{b}	7.24±0.05^{c}	7.08±0.05^{d}
Alkalinity	795.00±8.66^{ab}	799.00±27.28^{a}	787.10±28.56^{ab}	765.40±24.62^{b}
CO_2	10.30±0.57^{d}	24.16±3.21^{c}	40.33±5.24^{b}	56.33±7.57^{a}
TAN	0.00±0.00	0.23±0.03^{a}	0.23±0.06^{a}	0.27±0.03^{a}
NH_3-N	0.0000±0.0000	0.0025±0.0003^{a}	0.0014±0.0004^{b}	0.0012±0.0002^{b}

DO (Dissolved Oxygen) = mg O_2 L^{-1}, Alkalinity = mg $CaCO_3$ L^{-1}, CO_2 = mg CO_2 L^{-1},TAN = NH_4^+ + NH_3-N mg L^{-1} , NH_3-N = mg L^{-1}.

Table 2: Glucose, osmolality, hematocrit, hemoglobin, lactate and pO_2 (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) transported during 8 h at different stocking densities with 1 mg $NaHCO_3$ L^{-1}. Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood Parameter	**Density (g L^{-1})**	**Before Transport**	**Time After Transport (h)**		
		Control	**0**	**2**	**24**
		51.7±8.7			
Glucose	15		$84.3±19.3^{zA}$	$87.7±15.4^{zA}$	$43.0±8.3^{B}$
(mg dL^{-1})	24		$195.4±48.9^{*yA}$	$161.8±55.4^{*yA}$	$68.6±44.4^{B}$
	33		$374.6±138.6^{*xA}$	$370.3±75.8^{*xA}$	$41.0±3.5^{B}$
		369.4±12.9			
Osmolality	15		$369.0±13.3^{y}$	$358.2±6.2^{y}$	362.0±16
(mOsm kg^{-1})	24		$392.1±12.4^{xyA}$	$369.4±8.9^{xyB}$	$379.3±20.9^{AB}$
	33		$411.0±44.7^{*x}$	$426.8±35.7^{*x}$	376.0±33.4
		27.0±4.7			
Hematocrit	15		28.3±3.3	23.6±2.1	22.9±5.9
(%)	24		32.9±5.6	25.8±4.6	26.5±6.7
	33		27.7±3.9	22.6±2.1	26.3±4.0
		8.1±3.4			
Hb	15		9.5±1.0	$8.0±0.7^{xy}$	8.2±2.1
(mmol L^{-1})	24		$11.2±1.9^{*}$	$8.8±1.6^{x}$	9.0±2.3
	33		9.4±1.3	$5.4±2.4^{y}$	8.9±1.4
		3.6±0.8			
Lactate	15		$3.5±0.6^{yA}$	$3.3±0.3^{yA}$	$2.1±0.5^{B}$
(mmol L^{-1})	24		$4.9±1.4^{yA}$	$3.4±0.6^{yB}$	$2.5±0.9^{B}$
	33		$6.0±2.9^{xAB}$	$11.2±8.2^{*xA}$	$1.9±1.2^{B}$
		5.2±2.5			
pO_2	15		5.2±2.3	2.7±1.2	5.7±3.3
(mm Hg)	24		3.8±2.3	4.6±3.3	4.2±2.7
	33		2.8±1.6	4.8±3.4	3.2±1.3

Table 3: Blood pH, pCO_2 and HCO_3^- (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) transported during 8 h at different stocking densities with 1 mg $NaHCO_3$ L^{-1}. Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood Parameter	**Density (g L^{-1})**	**Before Transport**	**Time After Transport (h)**		
		Control	**0**	**2**	**24**
		7.2±0.1			
pH	15		$7.5\pm0.1^{*A}$	7.2 ± 0.1^{xB}	7.2 ± 0.1^{C}
	24		$7.5\pm0.1^{*A}$	7.1 ± 0.1^{yB}	7.1 ± 0.2^{B}
	33		$7.6\pm0.1^{*A}$	$7.3\pm0.1^{*xB}$	7.2 ± 0.1^{B}
		18.1±4.2			
pCO_2	15		$36.1\pm6.4^{*zA}$	$9.5\pm0.5^{*yB}$	$9.1\pm2.2^{*B}$
(mm Hg)	24		$51.1\pm9.7^{*yA}$	12.4 ± 2.2^{xyB}	11.9 ± 4.5^{B}
	33		$68.8\pm14.9^{*xA}$	14.3 ± 4.5^{xB}	9.5 ± 1.8^{B}
		6.4±1.0			
HCO_3^-	15		$25.8\pm3.7^{*yA}$	4.5 ± 0.7^{xyB}	3.4 ± 0.6^{B}
(mmol L^{-1})	24		$38.6\pm5.3^{*yA}$	4.3 ± 0.8^{yB}	3.6 ± 0.7^{B}
	33		$62.9\pm18.2^{*xA}$	7.9 ± 3.1^{xB}	3.5 ± 0.5^{B}

Capítulo 2

Transport of juvenile cobia *Rachycentron canadum*: combined effects of salinity and temperature

Artigo nas normas de submissão da revista Aquaculture.

Abstract

This experiment aimed to study the water quality and the stress response of juvenile cobia transported at different salinity and temperature combinations (S12-T19, S30-T19, S12-T23 and S30-T23), all in triplicate. Fish (29.6 g) were stoked in polyethylene bags at a density of 27 g L^{-1} and transported for 8 h. Blood samples were taken for secondary stress response analysis before transportation (Control), 0 (immediately after transport), 2 and 24 h afterwards. Water samples were collected to measure temperature, salinity, oxygen, pH, alkalinity, total and unionized ammonia and to calculate CO_2. Salinity was decisive for survival, there was no mortality for cobia when transported at salinity 12, independent of temperature. On the other side, at the higher salinity (30) there was mortality at T19 (1.2%) and T23 (7.4%). Blood glucose increased for fish in all treatments immediately after transport. However, after 24 h, glucose returned to the initial levels. Blood hypercapnia was observed immediately after transport for all treatments, blood acidification was overcome by significant elevation of blood HCO_3^-, which actually resulted in blood pH rise. Despite of no mortality at S12-T23, it was observed the highest CO_2 concentration in the water (81 mg CO_2 L^{-1}) at this treatment. According to the present results, it is safe to use the combination of salinity 12 g L^{-1} and temperature 19°C to transport juvenile cobia in stocking densities up to 27 g L^{-1}.

Keywords: Transportation, water quality, blood acidification, blood parameters.

1. Introduction

Cobia *Rachycentron canadum* is an important species for aquaculture, with a production of more than 40 thousand tons in 2014. The main producing countries are Taiwan, China, Vietnam and Panamá and they are most cultured in cages (Benetti et al., 2010; FAO, 2016). Its optimal growth at 27°C and salinity 30 g L^{-1}, and its isosmotic point is equivalent to 11.2 g L^{-1} (Sun et al., 2006; Burkey, et al., 2007; Chen et al., 2009). Cobia culture faces some problems that include high mortality during transport from nursery to grow-out cages, so the weight proposed for its transport is nearly 30 g (Liao et al., 2004).

Closed system is a widespread method to transport fish. Some features of closed system include reduction on the volume of water transported and the advantage of being

more economical (Berka, 1986). However, closed systems have disadvantages mainly about water quality, by decreasing dissolved oxygen and pH, and increasing ammonia and carbon dioxide concentrations. Changes in the concentrartions of these substances leads to a poor water quality in closed systems that activates a cascade of physiological responses to cope with the stress caused (Amend et al., 1982; Golombieski et al., 2003). One of the side effects of low water pH is blood acidification, which impairs blood oxygenation due to the Root and Bohr effects (Claiborne et al., 1999).

A stressful condition may lead to increased gill permeability, thus resulting in higher water gain and blood ion loss for freshwater fish. Marine fish face an even higher loss of water and ion influx than usual and therefore an osmoregulatory disturbance is observed (Wendelaar Bonga, 1997; Harmon et al., 2009). Increasing water salinity for freshwater fish or reducing it for marine fish during transport, favors a gradient decline between water and fish blood, which may minimize energy expenditure for osmoregulation, and consequently, decrease the stress response (Lim et al., 2003). Some freshwater fish transported in low salinity water (≈ 3 g NaCl L^{-1}) presented a reduction in the stress responses (Urbinati and Carneiro, 2006; Gomes et al., 2006; Brandão et al., 2008; Oyoo-Okoth et al., 2011; Tacchi et al., 2015). However, little is known for the transport of marine fish at lower salinity, i.e. close to the isosmotic point (Weirich and Tomasso, 1991; Stiegletz et al., 2012). Cobia (1.65 g) transported for 24 h at 19°C under different stocking densities (5 – 20 g L^{-1}) and salinities (12 and 32 g L^{-1}) presented a better survival at the highest stocking densities (up to 15 g L^{-1}) when transported at the lowest salinity (12 g L^{-1}) (Stieglitz et al., 2012).

Higher temperatures increase metabolic rates, so lowering temperature (within specific limits according to each species) is advisable during fish transport (Ross and Ross, 2008). The reduced metabolism due to the lower water temperature favors preservation of water quality, thus improving overall fish condition (Golombieski et al., 2003; Harmon, 2009). Colburn et al. (2008) transported cobia (1.5 – 3 g) at different loading densities (5 – 25 g L^{-1}) and temperatures (19 – 25°C). Mortalities after transport were similar for both temperatures (2.2%), so the authors indicated that cobia can be transported for 24 h with low mortality at densities not exceeding 20 g L^{-1} at temperatures between 19 and 25°C.

Although Stieglitz et al. (2012) and Colburn et al. (2008) transported cobia at different salinity and temperature conditions, blood parameters were not accessed due to

the small size of their fish (1-3 g). Other than that, Liao et al. (2004) recommended that cobia ought to weigh at least 30 g prior to be stocked in cages. Therefore, this study aimed to analyze water quality, survival, and secondary stress responses of larger juvenile cobia (30 g) transported at different salinity (12 and 30 g L^{-1}) and temperature (19 and 23°C) combinations.

2. Material and methods

2.1 Animals

Juvenile cobia were purchased from a commercial hatchery and were reared at Laboratory of Marine Fish Culture (LAPEM) at Federal University of Rio Grande (FURG) in a recirculating aquaculture system (temperature 26.5°C; salinity 30 g L^{-1}) until the trials. They were fed twice daily with a commercial diet (57% crude protein; 14.5% lipid - NRD, INVE, Grantsville, UT, USA), and food was withheld 24 h prior to the experiment. This experiment was approved by Ethical and Animal Welfare Committee of the Federal University of Rio Grande – FURG (Certificate number 020/2014).

2.2 Transport

Juvenile cobia (29.6±4.6 g; 18.4±0.8 cm) were placed in polyethylene bags filled with 10 L of water, 20 L of pure oxygen, buffered with 1 g $NaHCO_3$ L^{-1}. Four treatments were defined as a combination of two salinities (12 or 30 g L^{-1}) and temperatures (19 and 23°C), further on described as: S12-T19, S30-T19, S12-T23, and S30-T23, all in triplicate. Also, three control bags for water quality were transported without fish. Salinity was reduced to 12 g L^{-1} by diluting seawater (30 g L^{-1}) with dechlorinated tap water (0 g L^{-1}). Temperature in the RAS was reduced to 19ºC and 23ºC within the last three hours before fish were packed. The bags were placed in styrofoam boxes and then were transported for 8 h. The final stocking density was 27 g L^{-1}.

Water samples from all bags were collected after transport. Handheld meters were used to measure oxygen and temperature with YSI Model 550A meter, (Yellow Springs Instruments, Yellow Springs, OH, USA), and pH with FE20-FiveEasyTM (Mettler Toledo, Switzerland). Alkalinity was measured following APHA (1999) and CO_2 was calculated with the software CO_2 Analysis Salt® (Timmons and Ebeling, 2010). TAN was determined accordingly to Solorzano (1969) and NH_3-N was calculated using the equations of Ostrensky et al. (1992) adapted from Whitfield (1974).

2.3 Sampling

Blood was sampled from the caudal vein with a heparinized 1 mL syringe. Three fish were sampled from three bags per treatment immediately after transport (0 h), and three fish from the remaining bags were placed in recovering tanks (one tank for each bag) filled with 50 L of water in the same salinity and temperature as they were transported and were sampled 2 and 24 h later. During the recovery period, temperature was increased to 25.5°C. Blood of nine fish was sampled before transport and used as a control. An I-STAT Portable Clinical Analyzer (Abbott Laboratories, Chicago, IL, USA) was used with along with a $CG8^+$ cartridge to measure glucose, hemoglobin, sodium (Na^+), potassium (K^+), ionized calcium (Ca^+), pH, partial gas pressure of CO_2 (pCO_2), displaying calculated values of blood bicarbonate (HCO_3^-) and O_2 (pO_2). Values for pCO_2 and HCO_3^- were temperature-corrected to the experimental temperature according to the manufacture´s specifications. Effectiveness of I-STAT measurements has been proved for cobia (Rodrigues et al., 2015) and other fish species (Kristensen, et al., 2010; Paust et al., 2011; Barbas et al., 2016). Blood was centrifuged (10,192 × *g*) for 10 min. (4°C) for plasma osmolality measurements using a vapor pressure osmometer (Vapro 5520; Wescor, Inc.; Logan, Utah, USA) and hematocrit was determined centrifuging blood for 10 min. (16,128 × *g*) (Hematocrit Centrifuge H-240, Hsiang Tai Machinery Industry CO., Taiwan).

2.4 Statistical analyses

Water quality and blood parameters were analyzed by one-way ANOVA, followed by Tukey test when significant differences were observed. Dunnett test was

applied to identify data points that were significantly different from Control levels. All analyzes were performed with a minimal significant level of $p<0.05$. All values were presented as mean and standard deviation.

3. Results

There was no mortality at salinity 12 g L^{-1}, independent of temperature. Mortality was only observed at the treatments S30-T19 (1.2%) and S30-T23 (7.4%).

Water quality parameters in the control bags, transported without fish were: 15.3 mg O_2 L^{-1}, pH 7.7, 16.1 mg CO_2 L^{-1}, alkalinity 750.4 mg $CaCO_3$ L^{-1}, 0.02 mg NH_4^+ + NH_3-N L^{-1} and 0.0002 mg NH_3-N L^{-1}. Final water quality parameters for fish transported at different combinations of temperature and salinity are shown in Table 1. Dissolved oxygen was higher at S12-T19 (10.9 mg L^{-1}) and lower at S30-T23 (3.7 mg L^{-1}). For the treatments with the lowest temperature (19°C), pH was higher when compared to 23°C. Alkalinity was lower for treatments with the lowest salinity (12 g L^{-1}). CO_2 concentrations were higher at the treatment S12-T23 (81 mg L^{-1}) and lower at S30-T19 (57 mg L^{-1}). Total ammonia (TAN) was higher at the trials with the highest temperature (23°C). For unionized ammonia, the highest concentration was at the treatment S12-T23.

There was an increase in glucose levels for all treatments at 0 h in relation to Control, they remained high until 2 h, but they all returned to Control levels after 24 h. Right after transport, the highest glucose values were for fish transported at S30-T23 when compared to other treatments. Hematocrit percentage decreased after 24 h for the treatments S12-T19 and S30-T23 in relation to Control. Hemoglobin concentration presented an elevation after transport at S12-T19, S12-T23 and S30-T19, but returned to Control levels after 2 h. There were no differences for lactate levels when compared to Control, but at 24 h the treatment S30-T23 was higher than all other treatments. There was no difference for blood oxygen when compared to Control, however at the treatment S12-T19, its concentration was lower at 2 h than at 24 h (Table 2).

Osmolality and Na^+ levels increased at 0 and 2 h compared to the control just for S30-T23, however they returned to basal levels after 24 h. There was a decrease at K^+ levels when compared to Control only right after transport for the treatment S12-T19,

but it returned to the control level after 24 h. There was a decrease in Ca^{+} levels at S12-T19 after 24 h in relation to Control (Table 3). All blood pH, pCO_2 and HCO_3^- levels increased for all treatments after transport (0 h), but they all returned for Control levels already at 2 h (Table 4).

4. Discussion

Salt addition and decreasing temperature are known practices used in aquaculture to improve survival and minimize stress response during freshwater fish transport, however these methods are less studied for marine fish (Harmon, 2009; Stieglitz et al., 2012). In this experiment, isosmotic salinity and reduced temperature contributed for cobia survival and reduction of stress response.

The dissolved oxygen was equal to, or above 7.6 mg O_2 L^{-1} at the lowest temperature, and below 5.5 mg O_2 L^{-1} at the highest one, independent of the salinity. Jundiá *Rhamdia quelen* (1-2.5 g) transported for 24 h (168 g L^{-1}) at different temperatures (15, 20 and 25ºC) showed highest dissolved oxygen at 15°C (18 mg L^{-1}) and lowest at 25°C (0.44 mg L^{-1}), which reflected in higher mortality (15%) at the highest temperature (Golombieski et al., 2003), showing the importance of this parameter during transport.

At higher densities, carbon dioxide (CO_2) concentrations could rise due to the higher oxygen consumption, so increase in CO_2 levels are usually related with low dissolved oxygen concentrations (Wurts and Durborow, 1992). In fact, the highest CO_2 concentrations were found at the treatments with the lowest dissolved oxygen (S12-T23 and S30-T23). Hypercapnia (73.2 mg CO_2 L^{-1}) and hypoxia (3.7 mg O_2 L^{-1}) were factors that probably contributed for the higher mortality (7.4 %) at the treatment S30-T23. The limit for CO_2 concentration for fish growth is considered 20 mg L^{-1} and concentrations above 40 mg L^{-1} are considered harmful for fish (Wedemeyer, 1996; Moran et al., 2008). Yellowtail *Seriola quinqueradiata* (1.4 kg) was exposed to different CO_2 concentrations (14 and 75 mg L^{-1} at 20°C), and all fish died after 8 h of exposure at the highest concentration (Lee et al., 2003), which was a lower concentration than that found in this study at the treatment S12-T23 (81.6 mg CO_2 L^{-1}), but no mortality was observed for cobia.

A higher CO_2 concentration interferes in pH levels, it is decreased due to the production of carbonic acid in the water (Wurts and Durborow, 1992). In this study,

despite the use of sodium bicarbonate, pH was reduced for all treatments, reaching the lowest level at the treatment S30-T23 (6.9), which is not considered harmful to cobia (Rodrigues et al., 2015).

Barbieri and Doi (2012) exposed cobia (19 g) to different ammonia concentrations (0-120 mg L^{-1} of TAN) at different salinities (5, 20, and 35 g L^{-1} / 25°C) and cobia was negatively affected with decreasing salinity: mortality and ammonia excretion were higher at 5 g L^{-1}. In this study, the highest total and unionized ammonia were at S12-T23 (3.2 and 0.015, respectively). So considering that lethal concentrations for both parameters for cobia are 38.5 mg L^{-1} of TAN and 1.13 mg NH_3-N L^{-1} (Rodrigues et al., 2007), the ammonia concentrations observed at this work were within safe levels.

Stress responses are affected by water quality, resulting in alterations in blood glucose, hematocrit, hemoglobin, lactate, oxygen uptake, osmolality, and ions, which are adaptatise reactions to stress conditions (Wendelaar Bonga, 1997). At higher temperatures, there is an increase in oxygen consumption and hyperglycemia is normally verified (Van Raaij et al., 1996; Golombieski et al., 2003). In this experiment, glucose levels were higher for all treatments right after transport especially for fish transported at S30-T23 (357 mg dL^{-1}) which was two-fold higher than at S12-T19 (173 mg dL^{-1}), but in all treatments, glucose levels returned to Control levels after 24 h. Differently from cobia, coho salmon *Oncorhynchus kisutch* (20 g) exposed to three different salinities (0, 10, and 28 g L^{-1}) did not present difference for glucose levels or oxygen consumption (Morgan and Iwama, 1998). Matrinxã *Brycon amazonicus* was also able to keep glucose similar to control levels when sodium chloride (6 mg L^{-1}) was added to the water (Urbinati and Carneiro, 2006).

During stressful situations, hematocrit and hemoglobin concentrations can increase in order to improve the oxygen transport (Wendelaar Bonga, 1997). In this study, hematocrit remained equal to Control right after transport, but had a slight decrease after 24 h for treatments S12-T19 and S30-T23. Sunshine bass (*Morone chrysops x Morone saxatilis*) were subjected to acute stress at different temperatures (5 – 30°C), and at 10°C there was an initial increase followed by a delayed decrease in hematocrit after 48 h from stress (Davis, 2004). Despite no changes found for hemoglobin at the treatment S30-T23, all other trials had an increase in hemoglobin concentration after transport, then returning to Control levels at 2 h. Although there was

no significant difference from Control, it was observed a slight decrease of blood pO_2 after transport, as a result of the increasing oxygen consumption due to the stress condition, which leads to a CO_2 accumulation in the blood, also observed in this study. Differently from hematocrit, hemoglobin concentration reacted by increasing its levels after transport, trying to maintain the gas transport, which was probably affected by Root and Bohr effects (Wendelaar Bonga, 1997; Claiborne et al., 1999).

Oxygen concentration and lactate levels are inversely correlated, since lactate is an energy source activated in hypoxia conditions (Wendelaar Bonga, 1997). Cobia apparently did not face an anaerobic situation, because despite the lower oxygen in the water for S12-T23 and S30-T23 (5.5 and 3.7 mg O_2 L^{-1} respectively), lactate concentrations after transport were not different between those treatments. This could be related again with the increase in hemoglobin concentration, which was able to cope oxygen blood transport and prevent lactate buildup (Olsen et al., 1995).

Only the treatment S30-T23 presented difference for osmolality among times by being higher after transport and at 2 h, but returning to Control levels after 24 h. Fish facing a stressful situation in a hyperosmotic medium tend to loose water and absorb electrolytes, thus raising their blood osmolality. On the other hand, fish gain water and loose electrolytes when in a hyposmotic medium (Robertson et al., 1988). Red drum *Sciaenops ocellatus* (40 – 140 g / 23-30 g L^{-1}) was transported (2.5 – 5.5 h/ 19.5 – 26°C) at different salinities (4 and 32 g L^{-1}), and fish transported at the lowest salinity showed a decrease in osmolality, while at the highest one there was an osmolality increase (Robertson et al., 1988). It is well known for some species that rearing fish in salinities near their isosmotic point can lead them to save energy from osmoregulatory process (Gaumet et al., 1995; Imsland et al., 2001). So cobia was able to keep blood plasma osmolality levels at the isosmotic condition (S12-T19 and S12-T23) and at hyperosmotic medium with lower temperature condition (S30-T19). It was observed a decrease in K^+ and Ca^+ concentrations for the treatment S12-T19 at 0 and 2 h, respectively. On the other hand, an increase was observed for Na^+ at S30-T23 for 0 and 2 h, however they were all restored after 24 h. During a stress situation, gill permeability increases and an ionic disturbance can occur (Postlethwaite and McDonald, 1995), as showed in this experiment.

High CO_2 levels in the water leads to blood hypercapnia, but pH decrease is controlled due to HCO_3^- release, establishing a CO_2/HCO_3^- equilibrium found in

homeostase (Claiborne and Heisler, 1986; Pelster, 2004). This equilibrium was noticed in this experiment, when right after transport (0 h) there was an increase in pCO_2 followed by a simultaneously increase in HCO_3^- and pH, that was restored already at 2 h. Jundiá (420 g) transported for 6 h (274 g L^{-1}/ 27°C) with extract of *Lipia alba* (0, 30 and 40 mg L^{-1}) showed increased CO_2 and HCO_3^- concentrations for all treatments after transport, but pH levels were kept unaltered (Becker et al., 2016).

An enhanced stocking density with higher survival is economically interesting for aquaculture (Harmon, 2009). Transport of cobia has been done at lower stocking densities, Stieglitz et al. (2012) transported smaller cobia (1.65 g) for 24 h using a combination of low salinity (12 g L^{-1}) and temperature (19°C), obtaining survival above 80% when stocking density was up to15 g L^{-1}. Larger cobia (30 g) were successfully transported (100% survival) by Pedron et al. (2016) for 8 h in a lower stocking density (10 g L^{-1}) but at higher salinity (30 g L^{-1}) and temperature (22.5°C). Therefore, the manipulation of salinity and temperature can improve overall conditions of cobia transported at a higher stocking density (27 g L^{-1}).

5. Conclusion

Juvenile cobia (30 g) can be safely transported in plastic bags up to 8 h at the density of 27 g L^{-1} by using a combination of reduced salinity (12 g L^{-1}) and low temperature (19ºC), which resulted in minimized secondary stress responses and 100% survival. Although no mortality was observed at S12-T23, this treatment is not recommended due to the high CO_2 concentration in the water (81.6 mg CO_2 L^{-1}), which could actually be an important trigger for mortality occurrence.

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Tables:

Table 1: Water quality parameters measured immediately after 8 h transport (mean ± SD) of juvenile cobia *Rachycentron canadum* at different salinity and temperature levels (S12-T19, S30-T19, S12-T23 and S30-T23). Different letters indicate significant differences ($p<0.05$) among treatments, as indicated by one-way ANOVA and Tukey's test.

Parameters	**Salinity and Temperature**			
	S12-T19	S12-T23	S30-T19	S30-T23
DO	10.9 ± 2.7^{a}	5.5 ± 1.5^{bc}	7.6 ± 1.6^{b}	3.7 ± 1.5^{c}
pH	7.1 ± 0.05^{a}	7.0 ± 0.04^{b}	7.1 ± 0.03^{a}	6.9 ± 0.04^{b}
Alkalinity	712.7 ± 18.2^{b}	711.3 ± 18.7^{b}	778.3 ± 19.3^{a}	774.4 ± 21.7^{a}
CO_2	65.2 ± 7.2^{b}	81.6 ± 8.1^{a}	56.8 ± 3.7^{c}	73.2 ± 6.3^{b}
TAN	2.4 ± 0.38^{b}	3.2 ± 0.4^{a}	2.2 ± 0.3^{b}	2.9 ± 0.4^{a}
NH_3-N	0.0119 ± 0.0018^{b}	0.0152 ± 0.0012^{a}	0.0093 ± 0.0009^{c}	0.0107 ± 0.0011^{bc}

DO (Dissolved Oxygen) = mg O_2 L^{-1}, Alkalinity = mg $CaCO_3$ L^{-1}, CO_2 = mg CO_2 L^{-1},TAN = NH_4^+ + NH_3-N mg L^{-1} , NH_3-N = mg L^{-1}.

Table 2: Glucose, hematocrit, hemoglobin, lactate and pO_2 (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) after 8 h of transport at different salinity and temperature levels (S12-T19, S30-T19, S12-T23 and S30-T23). Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood	**S x T**	**Before transport**	**Time after transport (h)**		
		Control	0	2	24
		40.7±9.8			
Glucose	S12-T19		173.5±64.4^{*yA}	142.2±23.9^{*yzA}	44.1±6.5^{yB}
(mg dL^{-1})	S12-T23		216.3±65.9^{*yA}	194.2±49.6^{*yA}	48.4±3.0^{xyB}
	S30-T19		195.0±66.3^{*yA}	120.0±36.1^{*zB}	49.3±7.4^{xyC}
	S30-T23		357.0±81.1^{*xA}	267.3±59.4^{*xA}	52.7±6.2^{xB}
		33.4±4.8			
Hematocrit	S12-T19		37.1±3.7^{xA}	30.5±4.0^{B}	25.4±4.2^{*C}
(%)	S12-T23		36.1±7.5^{x}	30.7±4.2	29.3±5.4
	S30-T19		38.5±5.4^{xA}	32.0±5.9^{AB}	28.6±8.2^{B}
	S30-T23		28.2±1.8^{y}	31.1±5.1	26.0±5.2*
		6.68±1.18			
Hb	S12-T19		9.47±2.03^{*A}	9.19±1.77^{A}	6.26±1.11^{B}
(mmol L^{-1})	S12-T23		11.48±2.08^{*A}	7.13±2.02^{B}	7.54±1.71^{B}
	S30-T19		10.98±2.05^{*A}	7.51±1.46^{B}	7.25±1.57^{B}
	S30-T23		8.35±3.61	7.55±0.96	7.27±1.10
		3.7±0.8			
Lactate	S12-T19		3.3±3.9	3.5±1.2	2.5±0.6^{y}
(mmol L^{-1})	S12-T23		4.9±1.6^{A}	3.8±2.2^{AB}	2.9±0.5^{xyB}
	S30-T19		2.7±1.8	3.2±1.1	2.5±0.9^{y}
	S30-T23		7.4±6.3	6.6±5.9	3.4±0.4^{x}
		12.5±10.7			
pO_2	S12-T19		5.9±3.1^{AB}	3.6±2.9^{B}	12.6±10.7^{A}
(mm Hg)	S12-T23		6.1±3.4	6.8±6.7	7.3±6.2
	S30-T19		5.0±2.9	5.9±8.7	6.7±5.3
	S30-T23		4.8±3.3	4.5±3.8	9.6±9.3

Table 3: Plasma osmolality, blood Na^+, K^+ and Ca^+ (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) after 8 h of transport at different salinity and temperature levels (S12-T19, S30-T19, S12-T23 and S30-T23). Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood	**S x T**	**Before transport**	**Time after transport (h)**		
		Control	0	2	24
		368±9.2			
Osmolality	S12-T19		353.2±19.66^{z}	373.7±24.7^{y}	359.4±5.8
(mmol kg^{-1})	S12-T23		395.7±38.9^{yA}	384.5±20.1^{xyAB}	355.1±12.4^{B}
	S30-T19		363.5±11.6^{yz}	365.7±15.6^{y}	354.3±9.5
	S30-T23		434.5±34.5^{*xA}	408.6±24.9^{*xA}	356±12.2^{B}
		174.3±3.6			
	S12-T19		157.2±5.6^{zC}	174.2±4.1^{A}	166.3±7.8^{B}
Na^+	S12-T23		164.5±2.4^{yB}	171.1±8.3^{A}	171.6±3.6^{A}
(mmol L^{-1})	S30-T19		167.2±3.8^{y}	169.5±3.8	171.5±5.4
	S30-T23		177.3±7.2^{*x}	176.7±5.8*	172.2±1.9
		5.14±0.72			
K^+	S12-T19		3.95±0.54^{*yB}	5.05±0.85^{A}	4.67±0.49^{AB}
(mmol L^{-1})	S12-T23		4.98±0.78^{x}	4.96±0.87	4.84±0.37
	S30-T19		4.27±0.68^{xy}	4.76±0.75	4.56±0.59
	S30-T23		5.05±0.21^{xy}	4.65±0.58	4.81±0.59
		1.31±0.43			
Ca^+	S12-T19		1.14±0.23	1.05±0.22	1.00±0.32*
(mmol L^{-1})	S12-T23		1.07±0.29	1.09±0.53	1.13±0.43
	S30-T19		1.26±0.31	1.04±0.41	1.12±0.38
	S30-T23		0.90±0.33	1.17±0.35	1.02±0.29

Table 4: Blood pH, pCO_2 and HCO_3^- (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) after 8 h of transport at different salinity and temperature levels (S12-T19, S30-T19, S12-T23 and S30-T23). Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood	**S x T**	**Before transport**	**Time after transport (h)**		
		Control	0	2	24
		7.18 ±0.07			
pH	S12-T19		$7.55\pm0.05^{*xA}$	7.09 ± 0.09^{B}	7.13 ± 0.08^{B}
	S12-T23		$7.54\pm0.07^{*xyA}$	7.18 ± 0.09^{B}	7.16 ± 0.09^{B}
	S30-T19		$7.46\pm0.07^{*yA}$	7.09 ± 0.09^{B}	7.18 ± 0.09^{B}
	S30-T23		$7.62\pm0.06^{*xA}$	7.20 ± 0.09^{B}	7.14 ± 0.09^{B}
		15.2 ± 2.1^{B}			
pCO_2	S12-T19		$43.1\pm8.5^{*yA}$	13.9 ± 4.8^{B}	10.1 ± 2.0^{B}
(mm Hg)	S12-T23		$65.8\pm10.1^{*xA}$	11.2 ± 3.4^{B}	10.3 ± 2.1^{B}
	S30-T19		$44.4\pm9.5^{*yA}$	9.7 ± 1.3^{B}	10.1 ± 2.7^{B}
	S30-T23		$59.3\pm10.1^{*xA}$	14.2 ± 4.6^{B}	10.6 ± 2.6^{B}
		5.7 ± 0.6^{B}			
HCO_3^-	S12-T19		$37.3\pm7.2^{*xA}$	4.2 ± 1.3^{xyB}	3.5 ± 1.1^{B}
(mmol L^{-1})	S12-T23		$56.2\pm5.5^{*yA}$	4.1 ± 1.3^{xyB}	3.7 ± 0.8^{B}
	S30-T19		$31.3\pm2.6^{*xA}$	3.0 ± 0.6^{xB}	3.8 ± 0.9^{B}
	S30-T23		$61.3\pm13.9^{*yA}$	5.8 ± 2.6^{yB}	3.6 ± 0.6^{B}

Transport of juvenile cobia *Rachycentron canadum*: effects of salinity and temperature

Highlights

- Cobia transported in closed system for 8 h presented no mortality at an isosmotic condition, neither at 19 nor at 23°C.
- However, at a hyperosmotic medium, mortality reached 1.2 % at 19°C and 7.4% at 23°C.
- Homeostase was restored after 24 h from transport.

Capítulo 3

Anesthesia and transport of juvenile cobia *Rachycentron canadum* using menthol

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Summary

This study determined the lowest effective concentration of menthol as anesthetic and also its suitability to minimize stress during transport of juvenile cobia *Rachycentron canadum*. Efficacy of menthol as anesthetic was tested at three concentrations: 35, 45, and 55 mg L^{-1}. Regarding the use of menthol to improve overall conditions of cobia during transport, they were transported for 8 h at three concentrations (0, 2.25, and 5.50 mg L^{-1}) at a density of 26 g L^{-1}. Mortality was not observed in both tests. Full anesthesia was achieved in less than 3 min and cobia recovered within 4 min when menthol concentration was 55 mg L^{-1}. For transport, there were no significant differences for water quality parameters using menthol. Blood glucose was elevated for fish transported with or without anesthetic on arrival. However, after 24 h, glucose had returned to control levels in all treatments. According to these results, menthol is recommended to anesthetize juvenile cobia at 55 mg L^{-1}, however its use during transport is not necessary, since the immediate stress response was not mitigated.

1 INTRODUCTION

The use of anesthetics is widespread in aquaculture practices to mitigate stress responses due to fish management, including weight/length measurement, tagging, blood sampling, vaccination, artificial reproduction, and transportation (Zhao et al., 2014). The ideal anesthetic should induce deep anesthesia within 3 min. and fish should recover normal swimming behavior in less than 5 min (Marking & Meyer, 1985). MS-222 and eugenol were tested for small (5-14 g) and large (297 g) cobia *Rachycentron canadum*, and the suggested concentrations to anesthetize the smallest were 60 mg MS-222 L^{-1} and 20 mg eugenol L^{-1}, while 150 mg MS-222 L^{-1} and 60 mg eugenol L^{-1} were

recommended for the largest (Gullian & Villanueva, 2009; Trushenski, Bowzer, Bowker, & Schwarz, 2012).

Menthol is the main component of the essential oil extracted from plants of the genus *Mentha*, and it is an important commodity for pharmaceutical and food industry (Eccles, 1994). Due to its anesthetics properties, acting by a voltage-dependent block of neuronal sodium channels and modulating $GABA_A$ (gamma-Aminobutyric acid) receptors, menthol has been used for marine invertebrates and fish anesthesia (Kasai, Hososhima, & Yun-Fei, 2014). Menthol is classified as a topical analgesic for humans by Food and Drugs Administration (FDA-USA), however its use has not been regularized for use in food fish (Eccles, 1994; Trushenski et al., 2013). Recently, menthol has been used as a fish anesthetic for several species like Nile tilapia (*Oreochromis niloticus*), Japanese medaka (*Oryzias latipes*) and fat snook (*Centropomus parallelus*) (Simões & Gomes, 2009; Kasai et al., 2014; Sepulchro, Carvalho, & Gomes, 2016), but its use has not been evaluated for cobia yet.

Fish transport is a stressful activity. It can induce several physiological and behavioral responses, leading to death if preventive measures are not applied, including the use of proper stocking density, adequate water quality (Golombieski, Silva, Baldisserotto, & Silva, 2003). Anesthetics have also been considered to minimize stress responses during fish transport, among them MS-222 (Zhao et al., 2014), benzocaine and eugenol (Navarro et al., 2016), lidocaine (Park et al., 2009), and the essential oils of *Lippia alba* (Becker et al., 2012), *Ocimum gratissimum* (Benovit et al. 2012), and *Spilantes acmella* (Barbas, Stringhetta, Garcia, Figueiredo, & Sampaio, 2016) have been used successfully. However, the responses to the use of anesthetics during transport can be varied. The essential oil of *Aloysia gratissima* (90 and 130 mg L^{-1})

caused mortality of juvenile flounder *Paralichthys orbignyanus* transported for 7 h (Benovit et al., 2012).

Cobia weighing 1.5 – 3 g have been transported with different densities, temperatures and salinities (Colburn, Walker, Berlinsky, & Nardi, 2008; Stieglitz, Benetti, & Serafy, 2012). However, Liao et al. (2004) suggested that cobia ought to weigh at least 30 g prior to be stocked in cages. Pedron et al. (2016) transported cobia weighing 30 g with benzocaine (0, 2, and 6 mg L^{-1}) and no benefits were observed. In fact, fish presented elevated stress responses when this anesthetic was employed. Therefore, the aim of this study was to determine the minimum effective concentration of menthol as anesthetic for juvenile cobia and to evaluate its effectiveness on reduction of the stress responses during transport of juvenile cobia (> 30 g) in a closed system.

2 MATERIALS AND METHODS

2.1 Animals

Juvenile cobia were reared at the Laboratory of Marine Fish Culture (Federal University of Rio Grande – FURG) where these experiments were performed. They were obtained from a natural spawning and then reared in a recirculating aquaculture system (RAS). After weaning, cobia were maintained at 26.5°C and salinity 30 g L^{-1}, while fed twice daily with a commercial diet, containing 57% crude protein and 14.5% lipid (NRD, INVE, Grantsville, UT, USA). The Ethical and Animal Welfare Committee of the Federal University of Rio Grande – FURG approved the experiments (Certificate number P073/2016).

2.2 Experiment 1: Determining the lowest effective concentration of menthol for cobia

The efficacy of menthol as anesthetic for cobia was tested at three concentrations: 35, 45, and 55 mg L^{-1}. A stock solution was prepared using menthol crystals (Vetec Química Fina Ltda., Brazil) by dissolving its content in 96% ethanol, in a proportion of 1:9 (menthol:ethanol, v:v).

Fish (46.3±7.7 g; 21.1±1.7 cm) were fasted for 24 h before the trial. The test was carried out in two 40 L glass aquaria, filled with seawater at a temperature of 26.5°C and salinity 30 g L^{-1}, which were kept constant throughout the experiment. Ten fish were evaluated for each concentration and the water was exchanged for every concentration of each anesthetic. The times of induction and recovery from anesthesia were recorded with a stopwatch and were characterized according to Park et al. (2008) with modifications (Table 1).

Another nine fish (per concentration) were anesthetized until stage A3 was achieved using the same menthol concentrations described above. A negative control, where fish were exposed to 0.48 ml ethanol L^{-1} (the volume used with the highest menthol concentration) for 3 min was also used. Anesthetized and ethanol-exposed fish were transferred to 50 L tanks filled with anesthetic-free water and blood samples were collected after 1 and 24 h from anesthesia. Blood was also collected from fish sampled from the original holding tank, called the Pre-Anesthesia group. Fish were carefully collected from their tanks and for blood sampling they were not anesthetized. This procedure did not take longer than 30 s. Glucose was measured with a portable glucometer (AccuCheek Advantage, Roche Diagnostics®, Germany) and hematocrit was determined by centrifuging the blood for 10 min at 16,128 × g (Hematocrit Centrifuge H-240, Hsiang Tai Machinery Industry CO., Taiwan).

2.3 Experiment 2: Transport of cobia using menthol

Fish (44.0±8.4 g; 20.6±1.4 cm) were fasted for 24 h before transport. Juvenile cobia were placed in 18 polyethylene bags (nine bags were used for each treatment). Each bag (60 L total volume) was filled with 10 L of water, 20 L of oxygen and 1 g L^{-1} of $NaHCO_3$ (pH buffer). The menthol concentrations used for transport were based on the results of the Experiment 1, where the lowest effective concentration was 55 mg L^{-1}. The treatments were 5 and 10% of 55 mg L^{-1} (2.25, and 5.5 mg L^{-1}) plus a group transported without anesthetic.

The stocking density during transport was 26 g L^{-1}. The salinity was reduced to 12 g L^{-1} to keep cobia close to an isosmotic condition (Burkey, Young, Smith & Tomasso, 2007; Stieglitz et al., 2012). The bags were packed in styrofoam boxes to maintain the temperature stable throughout the transport (18.4 ± 0.1ºC). Temperature in the RAS was reduced to 19ºC within the last 3 h (2.5°C per hour), before fish were packed to reduce fish metabolism (Colburn et al., 2008). The boxes were then transported by truck for 8 h. Three extra bags filled with water and oxygen (Without Fish) were packed and used as controls for water quality.

On arrival, water quality parameters were measured in all bags. Temperature and O_2 concentration were measured with an oximeter YSI Model 550A meter (Yellow Springs Instruments, Yellow Springs, OH, USA) and the pH was measured with a pH meter FE20-FiveEasy™ (Mettler Toledo, Switzerland). Alkalinity was measured following APHA (1999) and CO_2 concentration was calculated with the software CO_2 Analysis Salt® (Timmons & Ebeling, 2010). TAN was determined accordingly to Solorzano (1969) and NH_3-N concentration was calculated using the equations of Ostrensky, Marchiori, & Poersch (1992) adapted from Whitfield (1974).

Blood of the Control group (n=9) was sampled just before fish were packed. The blood of three fish sampled from three bags per treatment was collected immediately on arrival (0 h), and fish from the remaining bags were placed in recovering tanks (one tank for each bag) filled with 50 L of water (salinity: 12 g L^{-1}; 23°C) for blood collection at 24 h after transport. Blood was collected from the caudal vein with a heparinized syringe (1 mL). Blood parameters were evaluated using I-STAT Portable Clinical Analyzer (Abbott Laboratories, Chicago, IL, USA). The I-STAT analyzer was used with a $CG8^{+}$ cartridge measuring glucose, hematocrit, hemoglobin, pH, partial gas pressure of CO_2 (pCO_2), displaying calculated values of blood bicarbonate (HCO_3^-). Values for pCO_2 and HCO_3^- were corrected to the experimental temperature according to the manufacturer´s specifications. The efficacy of I-STAT measurements has been proved for cobia (Rodrigues, Pedron, Romano, Tesser, & Sampaio, 2015) and other fish species (Kristensen, Rosseland, Kiessling, Djordevic, & Massabau, 2010; Barbas et al., 2016).

2.4 Statistical analyses

All parameters were analyzed by one-way ANOVA, followed by Tukey test when significant differences were observed. Dunnett test was applied to identify data points that were significantly different from Control levels for blood parameters. All analyzes were performed with a minimal significant level of $p<0.05$. All values were presented as mean and standard deviation.

3 RESULTS

3.1 Experiment 1: Determining the lowest effective dose of menthol for cobia

There was no mortality during the experiment. All fish reached the three stages of anesthesia and recovery (Table 2). For induction time, fish exposed to 35 mg L^{-1} took a longer period to reach stage A1. Stage A3 was not reached within the 3 min limit for cobia exposed to 35 and 45 mg L^{-1}, only fish anesthetized with 55 mg L^{-1} reached stage A3 within the established time. For recovery, there was no significant difference among concentrations on time to reach all stages. Independent of concentration, all fish resumed swimming within the 5 min limit.

The blood parameters are shown in Table 3. After 1 h recovering from anesthesia, blood glucose in all treatments was significantly higher than Pre-anesthesia fish ($p<0.05$), except for those exposed to ethanol alone. After 24 h, glucose were back to the Pre-anesthesia level in all menthol concentrations ($p>0.05$). There was no significant difference for hematocrit among treatments ($p>0.05$).

3.2 Experiment 2: Transport of cobia using menthol

All fish survived throughout the transport.

Water quality parameters after 8 h of transport are shown in Table 4. Dissolved oxygen concentration at all treatments was significantly lower than the treatment Without Fish ($p<0.05$). On the other side, CO_2 concentrations were significantly higher in all treatments compared to Without Fish ($p<0.05$). There was no significant difference among treatments for alkalinity ($p>0.05$). After transport, there was no significant difference for pH among treatments ($p>0.05$), nevertheless pH levels were significantly lower than control at 0 and 5.50 mg L^{-1} ($p<0.05$). Ammonia (TAN and NH_3-N) significantly increased after fish transport ($p<0.05$), but there was no significant difference among cobia transported with or without menthol ($p>0.05$).

Glucose, hematocrit, hemoglobin, and pO_2 are shown in Table 5. On arrival, blood glucose of juvenile cobia was significantly higher than Before Transport ($P<0.05$). Blood glucose peaked 4 fold for juvenile cobia transported at 5.5 mg menthol L^{-1}. However, blood glucose returned to Control levels independent on the use of anesthetic or not 24 h after transport. Hematocrit and hemoglobin were also higher on arrival, and remained above Before Transport after 24 h ($p<0.05$), except for fish transported without anesthetic, which returned to the levels of Before Transport ($p>0.05$). On arrival, pO_2 concentrations were different from Before Transport, except for fish at 2.25 mg menthol L^{-1}, however all treatments presented lower pO_2 concentrations after 24 h, not returning to control levels.

Blood pH, pCO_2 and HCO_3^- are presented in Table 6. Significant alkalosis was observed immediately after transport, returning to Control levels only at 2.25 mg menthol L^{-1}. Increased levels were observed for pCO_2 and HCO_3^- right after transport, however all treatments were similar to Control after 24 h ($p>0.05$).

4 DISCUSSION

Fish welfare has received a great interest lately, and the study of anesthetics is an important topic, due to its ability to reduce stress response and injuries during common aquaculture practices or even during surgical interventions (Sneddon, 2012). In this study, cobia reached the final anesthesia stage in less than 3 min. only at 55 mg L^{-1}. Regarding recovery, all fish had resumed normal swimming behavior before 5 min., independent of menthol concentrations. The most effective concentration for short time handling fat snook *C. parallelus* (1.6 g) is 37 mg menthol L^{-1}, and 50 mg menthol L^{-1} is recommended for surgical interventions (Sepulchro et al., 2016). Further on, to Nile tilapia *O. niloticus* (14 g), concentrations between 150 and 200 mg menthol L^{-1} were

effective and safe to induce anesthesia for handling procedures (Simões & Gomes, 2009). Indeed, size/weight and fish species are factors that may affect the efficacy of an anesthetic (Ross & Ross, 2008).

Blood glucose has regularly been used as an indicator of stress in fish (Wendelaar Bonga, 1997), including cobia (Trushenski, Schwarz, Takeuchi, Delbos, & Sampaio, 2010). In this study, all concentrations of menthol caused an increase in glucose levels after 1 h from anesthesia. Nevertheless, after 24 h glucose levels were all similar to Pre-anesthesia. Due to hypoxia, anesthetized fish usually release catecholamines, and blood glucose rises as a consequence (Wendelaar Bonga, 1997), as seen in this study. This pattern was also observed for several anesthestics with different species, like MS-222, eugenol and benzocaine for cobia (Trushenski et al., 2012), clove oil for kelp grouper *Epinephelus bruneus* (Park et al., 2008) and MS-222 and clove oil for rainbow trout *Oncorhynchus mykiss* (Velisek et al., 2011).

Sedation during live fish transport can be a positive way to reduce fish metabolism, by reducing stress response and consequently maintaining good water quality, for short, or even long journeys (Ross & Ross, 2008). Closed systems (polyethylene bags filled with water and oxygen) have some limitations, as the build up of ammonia and CO_2, coupled to depletion of oxygen along transport (Golombieski et al., 2003).

In the present study, no mortality was found for cobia transported with or without menthol for 8 h. The use of pH buffers as sodium bicarbonate is encouraged because acid water could be lethal for fish during transport (Treasurer, 2012). The use of 1 g $NaHCO_3$ L^{-1} in this study was able to keep water pH levels above 7.47, considered safe for cobia. According to Rodrigues et al. (2015), only an acute exposure

to pH level below 6.5 could negatively affect cobia physiology and induce histopathology.

Oxygen transport to tissues can be reduced when CO_2 levels in the water are higher than 40 mg L^{-1}, which could induce hypercapnia, reducing the hemoglobin oxygen carrying capacity (Wedemeyer, 1996). In the present study, CO_2 levels during fish transportation did not differ among treatments, and the highest value was 28.8 mg CO_2 L^{-1} for cobia transported without anesthetic, lower than the upper limit (40 mg L^{-1}). On the other hand, 13 mg CO_2 L^{-1} was the highest CO_2 concentration found for cobia transported for 8 h in a density of 10 g L^{-1} with 2 mg benzocaine L^{-1} (Pedron et al., 2016). These results could elucidate the important relationship between stocking density and the release of CO_2 during transportation.

The acute toxic concentration (LC_{50}-96h) of ammonia to juvenile cobia is 1.13 mg N-NH_3 L^{-1}, or approximately 38.5 mg TAN L^{-1} (Rodrigues, Schwarz, Delbos, & Sampaio, 2007), then ammonia concentrations were within the safe level for cobia. Menthol was not able to reduce ammonia excretion for cobia, as total ammonia was similar for cobia transported with or without anesthetic, that reached 4.37 mg TAN L^{-1} at 5.5 mg menthol L^{-1}. The same was observed for fat snook *C. parallelus* transported with menthol (0, 3.7, and 7.4 mg L^{-1}) for 10 h (16 g L^{-1}), TAN values ranged from 3 to 6 mg TAN L^{-1}, suggesting menthol did not reduce metabolism of fat snook (Sepulchro et al., 2016).

Blood glucose peaked immediately after transport for all treatments, but they all were reduced after 24 h, reaching levels similar to Before Transport. Largemouth bronze gudgeon *Coreius guichenoti* transported for 14 h on a MS-222 bath (30 mg L^{-1}) also presented higher glucose levels after transport, although fish transported without anesthetic recovered after 4 h and fish transported with MS-222 recovered only seven

days later (Zhao et al., 2014). Anesthetics are used during transport to reduce metabolism and minimize the stress responses (Park et al., 2009; Becker et al., 2012; Benovit et al., 2012; Barbas et al., 2016; Navarro et al., 2016). Nonetheless, some studies indicate that an anesthetic itself could stimulate a higher stress response (Velisek et al., 2011; Pedron et al., 2016). Glucose levels of cobia were higher in the highest menthol concentration after transport, thus suggesting a possible stress induction due to the presence of menthol.

Fish submitted to stress are likely to have a higher hematocrit percentage owed to erythrocyte swelling and spleen contractions, which leads to an increase in hemoglobin concentration (Wendelaar Bonga, 1997). The hematocrit percentage and hemoglobin concentration were higher than Control for all treatments at 0 and 24 h after transport, except for cobia transported without anesthetic, that returned after 24 h. Pedron et al. (2016) transported cobia with benzocaine (2 and 6 mg L^{-1}) for 8 h and no differences were found among treatments for hematocrit. Hematocrit and hemoglobin have been widely measured as a stress secondary response for fish, however these responses seem to diverge depending on acute or chronic exposure to the stressor. Both hematocrit and hemoglobin are more likely to be affect in chronic stress conditions (Wandellar Bonga, 1997). However, the acute stress situation, fish showed an increase in hematocrit and hemoglobin when menthol was added and were not capable to recover to the basal levels after 24 h, indicating that cobia needs more time to fully recover. A result that corroborates with the increase in these hematological parameters is the decrease in blood pO_2 concentration after transport (0 and 24 h). Although the environment in the plastic bag had been in hyperoxia, blood pO_2 was reduced after transport and it did not recover within 24 h, and this could be related to the increased blood pCO_2. A heightened pCO_2 was observed in the blood after transport. The energy

expenditure to maintain homeostasis in stress situations results in increased oxygen consumption, which leads to a CO_2 release in the blood, exposing fish to Root and Bohr effect (Wendelaar Bonga, 1997; Claiborne, Edwards, & Morrison-Shetlar, 2002). Thus, hematocrit and hemoglobin levels tend to increase attempting to keep the transport of blood gases, since the blood oxygen carrying capacity is impaired due to the higher blood CO_2 levels (Claiborne et al., 2002). Petochi et al. (2011) observed the same pattern for European sea bass *Dicentrarchus labrax* (202 g), where blood pO_2 decreased and hematocrit and hemoglobin levels increased when fish were exposed to hypercapnia.

Reactions catalyzed by the enzyme carbonic anhydrase are responsible for CO_2 excretion and acid-base regulation. So, considering a hypercapnia situation, the absorption of bicarbonate is the main factor for pH recovery in marine teleosts (Claiborne et al., 2002). In this study, blood CO_2 levels were higher after transport, likewise pH and HCO_3^-. That was in accord with Salbego et al. (2015) that transported silver catfish *R. quelen* for 6 h with methanolic extract of *Condalia buxifolia* (5 and 10 mg L^{-1}) and also found a hypercapnia condition after transport, but blood HCO_3^- increased in order to compensate the higher CO_2 levels and avoid reducing blood pH.

According to the results of the present study, menthol (55 mg L^{-1}) is an effective anesthetic for cobia, inducing deep anesthesia safely. However, adding menthol up to 5.5 mg L^{-1} when transporting cobia in a closed system does not mitigate the stress responses, actually there is even an immediate higher stress response, compared to transport without the anesthetic. Nevertheless, it is safe to transport cobia at a stocking density up to 26 g L^{-1} for 8 h in a closed system at 18.5°C, as 100% survival is obtained.

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Tables:

Table 1: Stages of anesthesia induction and recovery in menthol efficacy tests for juvenile *Rachycentron canadum*. Modified from Park et al. (2008).

Stages	**Characteristic behavior**
Induction	
A1	Loss of equilibrium (sedation)
A2	Swimming stopped (deep anesthesia)
A3	Erratic opercular movement
Recovery	
R1	Normal opercular movement
R2	Swimming restarted
R3	Equilibrium recovered

Table 2: Anesthesia induction and recovery times (in seconds: mean ± SD) of juvenile cobia *Rachycentron canadum* (n=10) exposed to three concentrations of menthol. Different letters indicate significant differences among treatments for each stage, as indicated by one-way ANOVA and Tukey's test.

Menthol (mg L^{-1})	**Induction time (s)**			**Recovery time (s)**		
	Stage A1	Stage A2	Stage A3	Stage R1	Stage R2	Stage R3
35	37 ± 8^{x}	149 ± 31^{x}	266 ± 68^{x}	12±15	115±52	223±69
45	29 ± 5^{y}	149 ± 27^{x}	254 ± 48^{x}	17±19	137±64	236±68
55	24 ± 3^{y}	90 ± 11^{y}	165 ± 24^{y}	36±45	149±81	243±99

Table 3: Glucose levels and hematocrit percentage for juvenile cobia *Rachycentron canadum* (n=9) anesthetized with different concentrations of menthol at 1 and 24 h after anesthesia. Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood parameter	**Menthol (mg L^{-1})**	**Pre-anesthesia**	**Time after anesthesia (h)**	
			1	24
		43.6±11.9		
Glucose	35		$89.1±20.7^{*xyA}$	$43.0±6.0^{B}$
(mg dL^{-1})	45		$119.1±30.2^{*xA}$	$42.0±7.4^{B}$
	55		$123.5±41.3^{*xA}$	$36.2±4.7^{B}$
	0.48 ml ethanol L^{-1}		$64.0±11.6^{y}$	$39.1±5.2^{B}$
		26.8±3.7		
Hematocrit (%)	35		30.5±3.9	27.1±4.2
	45		28.6±6.6	25.8±4.3
	55		29.4±5.3	24.7±4.0
	0.48 ml ethanol L^{-1}		24.0±4.3	25.8±4.3

Table 4: Water quality parameters (mean ± SD) measured immediately after 8 h of transport of juvenile cobia *Rachycentron canadum* using menthol. Different letters indicate significant differences among treatments, as indicated by one-way ANOVA and Tukey's test. DO (Dissolved Oxygen) = mg O_2 L^{-1}; Alkalinity = mg L^{-1} as $CaCO_3$; CO_2 = mg CO_2 L^{-1}; TAN = mg L^{-1} NH_4^+ + NH_3^-; NH_3-N = mg NH_3-N L^{-1}.

Parameters			**Menthol (mg L^{-1})**	
	Without Fish	**0**	**2.25**	**5.50**
DO	22.26 ± 1.04^{x}	15.49 ± 3.21^{y}	13.83 ± 2.85^{y}	15.67 ± 1.42^{y}
CO_2	7.00 ± 0.00^{y}	28.80 ± 2.39^{x}	26.66 ± 3.44^{x}	28.66 ± 2.73^{x}
Alkalinity	678.88 ± 9.27^{y}	672.00 ± 9.1^{y}	675.00 ± 3.16^{y}	673.33 ± 10.32^{y}
pH	8.05 ± 0.01^{x}	7.47 ± 0.04^{y}	7.51 ± 0.06^{yx}	7.47 ± 0.05^{y}
TAN	0.09 ± 0.06^{y}	4.31 ± 0.41^{x}	4.09 ± 0.50^{x}	4.37 ± 0.33^{x}
NH_3-N	0.003 ± 0.002^{y}	0.037 ± 0.016^{x}	0.044 ± 0.006^{x}	0.043 ± 0.004^{x}

Table 5: Glucose, hematocrit, hemoglobin and pO_2 (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) transported with menthol. Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood parameter	**Menthol (mg L^{-1})**	**Before Transport**	**After Transport**	
		Control	0 h	24 h
		64.2±22.5		
Glucose	0		$171.4±27.1^{*yA}$	$48.7±10.0^{B}$
(mg dL^{-1})	2.25		$184.1±77.7^{*xyA}$	$49.9±25.5^{B}$
	5.50		$258.0±73.4^{*xA}$	$49.5±16.6^{B}$
		21.7±3.4		
Hematocrit (%)	0		$29.1±3.2^{*}$	26.7±5.3
	2.25		$30.4±3.4^{*}$	$28.0±5.6^{*}$
	5.50		$32.6±3.2^{*}$	$32.1±3.5^{*}$
		7.4±1.2		
Hemoglobin	0		$9.9±1.1^{*}$	9.1±1.8
(mmol L^{-1})	2.25		$10.4±1.3^{*}$	$10.4±2.3^{*}$
	5.50		$11.1±1.1^{*}$	$10.9±1.2^{*}$
		7.5±4.1		
pO_2	0		$4.6±1.6^{*}$	$4.1±2.9^{*}$
(mm Hg)	2.25		$4.8±1.2^{A}$	$3.0±1.2^{*B}$
	5.50		$4.1±1.0^{*A}$	$2.5±1.4^{*B}$

Table 6: Blood pH, pCO_2 and HCO_3^- (mean ± SD) from juvenile cobia *Rachycentron canadum* (n=9) transported with menthol. Different lowercase letters indicate significant differences ($p<0.05$) among treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) for the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test. Asterisks (*) indicate significant differences ($p<0.05$) when compared to values Before Transport, as indicated by Dunnett test.

Blood parameter	**Menthol (mg L^{-1})**	**Before Transport**	**After Transport**	
		Control	0 h	24 h
		7.0±0.1		
pH	0		$7.5\pm0.1^{*A}$	$7.2\pm0.1^{*B}$
	2.25		$7.5\pm0.1^{*A}$	7.2 ± 0.2^{B}
	5.50		$7.5\pm0.1^{*A}$	$7.2\pm0.1^{*B}$
		5.5±0.6		
HCO_3^-	0		$27.7\pm4.5^{*yA}$	7.6 ± 0.8^{xB}
(mmol L^{-1})	2.25		$31.6\pm4.1^{*xyA}$	6.5 ± 0.6^{yB}
	5.50		$34.9\pm3.5^{*xA}$	7.1 ± 1.0^{xyB}
		20.3±2.8		
pCO_2	0		$38.4\pm7.4^{*A}$	17.8 ± 4.4^{B}
(mm Hg)	2.25		$39.8\pm4.5^{*A}$	18.7 ± 5.2^{B}
	5.50		$41.9\pm4.8^{*A}$	19.7 ± 2.8^{B}

Capítulo 4

Tricaine Methanesulphonate (MS-222) and Eugenol as Anesthetics for Juvenile Cobia *Rachycentron canadum*

Artigo submetido à revista Aquaculture International.

Abstract

This study evaluated the use of MS-222 and eugenol as anesthetics for juvenile cobia *Rachycentron canadum*. Cobia were anesthetized at four concentrations of MS-222 (60, 80, 100, and 120 mg L^{-1}) and eugenol (20, 30, 40, and 50 mg L^{-1}) to observe induction and recovery times. After that, fish were exposed to the three higher concentrations until deep anesthesia was achieved. Then, blood samples were collected after 1 and 24 h of recovery to measure glucose, osmolality and hematocrit. Pre-anesthesia status was also assessed. All fish achieved anesthesia within 3 min and recovered in less than 5 min, except those at 60 and 80 mg MS-222 L^{-1} and at 20 eugenol L^{-1}, which took longer to be anesthetized. Hyperglicemia was observed at all treatments after 1 h, although after 24 h all fish had returned to Pre-anesthesia levels. Hematocrit was unaltered for both anesthetics. Osmolality was also similar for all treatments 24 h after anesthesia. Therefore, MS-222 at 100 mg L^{-1} and eugenol at 30 mg L^{-1} were considered safe and effective concentrations and the best cost-benefit for juvenile cobia.

Running head: MS-222 and eugenol for cobia

Keywords: Anesthesia. Blood parameters. Rachycentridae. Stress response. Welfare.

Introduction

Anesthetics are currently used in the management of modern aquaculture during procedures such as transport, blood sampling, weight/length measurements, surgery, vaccination and other practices in order to facilitate handling and minimize the physiological stress responses in fish (Weber 2011). Among these responses, there are changes in plasma cortisol and catecholamines as a primary response. These effects induce secondary responses related to energy requirements, such as increased blood glucose levels and hemoglobin concentration, besides disturbance in the hydromineral balance. The tertiary response is related with the organism and population changes due to stress, having as consequences reduced growth, impaired reproduction and immune response, and may even lead to mortality (Wendelaar Bonga 1997; Bolasina 2006).

In order to minimize the stress response for fish while handling them, it is important to choose an anesthetic considering: product efficacy, cost, market availability, and ease and safety in handling (Marking and Meyer 1985). Furthermore, it is necessary to know the optimal concentration of an anesthetic, because inappropriate doses can cause mortality (Roubach et al. 2005).

Tricaine methanesulphonate (MS-222) is a synthetic local anesthetic and its effect is provided by the suppression of the nervous system, blocking the sodium neuronal channels, thus decreasing the nerve excitability (Carter et al. 2011). MS-222 is the only anesthetic approved for food fish by the Food and Drug Administration (FDA – USA) and Health Canada, but fish can only be consumed 21 days after being exposed to this anesthetic (Meinertz and Schreier 2009; Trushenski et al. 2013).

Eugenol is the active ingredient of clove oil, which is extracted from the stem, leaves and flowers of the *Syzygium aromaticum* tree (Javahery et al. 2012). Eugenol has

a combined effect with neurotransmitters implicated in pain, activating $GABA_A$ (gamma-Aminobutyric acid) and inactivating glutamate, which acts on N-metil-d-aspartate (NMDA) receptors. This anesthetic also blocks vanilloid receptors that are responsible for pain transmission (Yang et al. 2003; Guénette et al. 2007). Eugenol is FDA approved for use in humans, being used as an anesthetic in medicine and dentistry (Cho and Heath 2000). Nevertheless, it is not approved for food fish, although it can be used under Investigational New Animal Drug (INAD) authorization (Trushenski et al. 2013).

Cobia, *Rachycentron canadum*, is a coastal, pelagic and migratory fish that is widely distributed in the tropical and subtropical oceans, except on the Pacific and Atlantic European coast (Shaffer and Nakamura 1989). Cobia is produced mainly in Asia, but also in the Americas (Liao et al. 2004; Benetti et al. 2010; Sampaio et al. 2011), reported worldwide production is around 40,000 ton (FAO 2016). Despite the growing interest in its culture, there are few studies about stress responses for cobia in captivity (Cnaani and MacLean 2009; Trushenski et al. 2010; Trushenski et al. 2012). Eugenol and MS-222 have already been used as anesthetics for small (5-14 g) and large (297 g) cobia. Suggested concentrations to anesthetize small cobia at low temperature (23ºC) was 60 mg MS-222 L^{-1} and 20 mg eugenol L^{-1} (Gullian and Villanueva 2009). Larger cobia, at a higher temperature (27ºC) were anesthetized on 150 mg MS-222 L^{-1} and 60 mg eugenol L^{-1} (Trushenski et al. 2012). Therefore, the aim of this study was to determine the lowest effective concentrations of MS-222 and eugenol as anesthetics for juvenile cobia (50 g), evaluating time to reach and recover from anesthesia, plus measuring secondary stress responses associated to the use of both anesthetics.

Materials and methods

Animals

Juvenile cobia (1 g) were obtained from a commercial hatchery and were reared in a recirculating aquaculture system (RAS) at the Laboratory of Marine Fish Culture (Federal University of Rio Grande - Brazil) until they were used in the trials. They were fed twice per day with a commercial diet, containing 57% crude protein and 14.5% lipid (NRD, INVE, Grantsville, UT, USA). Temperature was 26.5°C and salinity 30 g L^{-1}

The experiments were approved by Ethical and Animal Welfare Committee of the Federal University of Rio Grande – FURG (Certificate number 020/2014).

Determining the lowest effective concentration of MS-222 and eugenol for cobia

The anesthetics used were MS-222 (Western Chemical Inc., Washington, USA) and eugenol (Biodinâmica, Brazil). Their efficacy was tested at four concentrations, as follows: MS-222 (60, 80, 100, and 120 mg L^{-1}) and eugenol (20, 30, 40, and 50 mg L^{-1}). Both anesthetics were prepared just before the experiments. Soluble in water, MS-222 was diluted directly in the aquarium and buffered with 1 g $NaHCO_3$ L^{-1}. Eugenol is not soluble in water, therefore a stock solution was prepared by dissolving its content in ethanol (96%), in a proportion of 1:9 (eugenol:ethanol, v:v).

Fish (53.6±9.4 g; 21.2±1.1 cm) were fasted 24 h before the trials and were exposed individually (n=10) at each concentration. Tests were carried out in 40 L glass aquaria filled with seawater (temperature 26.5°C and salinity 30 g L^{-1}). Water was exchanged for every concentration of each anesthetic. Once fish reached the deep

anesthesia stage, it was gently transferred to an anesthetic free aquarium in order to observe its recovery.

The stages of induction and recovery from anesthesia are described in Table 1. They were characterized and modified according to Park et al. (2008).

Table 1.

Evaluating secondary stress responses

The anesthesia procedures were the same as explained above. Three concentrations of each anesthetic were chosen to evaluate the secondary stress response: 80, 100, and 120 mg MS-222 L^{-1} and 30, 40, and 50 mg eugenol L^{-1}. All blood samples were collected not exceeding 30 s after fish were captured. The Pre-anesthesia status of cobia was assessed in undisturbed and non-anesthetized fish. Cobia were also exposed to 0.43 mL ethanol L^{-1} (the volume used with the highest eugenol concentration), for a period not exceeding 3 min (Marking and Meyer 1985). Fish were then transferred to 50 L tanks filled with anesthetic-free water and blood samples were collected 1 and 24 h after anesthesia to measure glucose, hematocrit and osmolality. Those fish were not anesthetized right before sampling to avoid anesthetic interference in the stress responses. Glucose was measured with a portable glucometer (AccuCheek Active, Roche Diagnostics®, Germany) and hematocrit was determined centrifuging blood for 10 min. at 16,128 x *g* (Hematocrit Centrifuge H-240, Hsiang Tai Machinery Industry CO., Taiwan). Blood was centrifuged (10,192 x *g*) for 10 min. (4°C) for plasma osmolality measurements using a vapor pressure osmometer (Vapro 5520; Wescor, Inc.; Logan, Utah, USA).

Statistical analyses

Induction and recovery times and blood parameters were analyzed by one-way ANOVA, followed by Tukey test when significant differences were observed. All analyzes were performed with a minimal significant level of $p<0.05$.

Results

Determining the lowest effective concentration of MS-222 and eugenol for cobia

No mortality was observed in this experiment.

Induction and recovery times from anesthesia for cobia exposed to MS-222 and eugenol are shown in Table 2. All fish survived exposure to MS-222 and eugenol, even at the highest concentrations tested.

Table 2.

Time to reach stage A1 among fish exposed to MS-222 was similar among all concentrations tested, except for 60 mg L^{-1}, that took a longer to show loss of equilibrium. Cobia reached stage A3 within 1 min when anesthetized with 120 mg MS-222 L^{-1}, which was over thirteen fold faster than at 60 mg MS-222 L^{-1} (837 s). Regarding recovery, when fish were anesthetized with 60 mg L^{-1}, they already presented behavioral characteristics of stage R1 when placed in the aquarium. There was no difference on time between fish exposed to 80 and 100 mg MS-222 L^{-1} to reach all stages of recovery. However, time to reach stage R3 for fish exposed to 60 and 120 mg MS-222 L^{-1} (88 and 101 s, respectively) were shorter than at 80 and 100 mg MS-222 L^{-1} (135 and 148 s, respectively).

For eugenol, deep anesthesia was achieved faster, as the concentration of eugenol increased, taking 197 s to anesthetize cobia at 20 mg eugenol L^{-1} and 64 s to anesthetize at 50 mg eugenol L^{-1}. Recovery time to stage R1 was similar for cobia exposed to 20, 30, and 40 mg eugenol L^{-1}, all faster than cobia anesthetized at 50 mg eugenol L^{-1}, which took 56 s to reestablish normal operculum movement. However, for full recovery, there was no difference among eugenol concentrations.

Evaluating secondary stress responses

There was also no mortality in this experiment.

Cobia exposed to MS-222 and eugenol showed hyperglicemia 1h after they were anesthetized. Glucose reached ≈ 100 mg dL^{-1} for cobia anesthetized with MS-222, concentration similar to that for cobia treated with eugenol (Table 3), independent of the concentrations tested. However, the glucose peak was higher for cobia exposed to ethanol alone, it reached 200.4 mg dL^{-1}, a 2-fold increase compared to cobia anesthetized with eugenol. Furthermore, after 24 h, glicemia returned to Pre-anesthesia levels for cobia in all treatments, including those exposed to ethanol.

Table 3.

Hematocrit did not show differences after 1 or 24 h from anesthesia for MS-222 and eugenol. However, there was a transient increase at 30 mg eugenol L^{-1} at 1 h (32.5%) (Table 4).

Table 4.

Osmolality levels of cobia anesthethized with MS-222 or eugenol were not different after 1 and 24 h from anesthesia for all concentrations tested. However, osmolality peaked at (390 mOsm L^{-1}) 1 h after exposure to ethanol (Table 5).

Table 5.

Discussion

Pain perception and long-term suffering has been identified for fish (Sneddon 2003; Braithwaite and Boulcott 2007; Sneddon et al. 2014). Hence, it is important to understand the role of different anesthetics in fish and describe the changes in physiological parameters due to their use (Guénette et al. 2007).

In this study, 100 and 120 mg MS-222 L^{-1} and 30, 40, and 50 mg eugenol L^{-1} achieved effectively the assumptions of Marking and Meyer (1985). Roubach et al. (2005) found 65 mg eugenol L^{-1} to be an efficient concentration to induce anesthesia in tambaqui *Colossoma macropomum*. Furthermore, for *Solea senegalensis* (65 g), the optimal MS-222 and eugenol concentrations were 75 and 30 mg L^{-1}, respectively (Weber et al. 2009).

According to Ross and Ross (2008), environmental (pH, temperature, salinity, and mineral content) and biological (genetic, stress, body condition, disease status, sex, and sexual maturity, size/weight and fish species) factors can affect the efficacy of anesthetics. Gullian and Villanueva (2009) recommended the use of 20 mg eugenol L^{-1} and 60 mg MS-222 L^{-1} (23°C) for biometry procedures on juvenile cobia (5 and 14 g). On the other hand, 150 mg MS-222 L^{-1} and 60 mg eugenol L^{-1} (27°C) were recommended to anesthetize larger cobia (297 g) (Trushenski et al. 2012). In the present study, intermediate size cobia (54 g) was anesthetized with 30 mg eugenol L^{-1} and 100 mg MS-222 L^{-1}. Comparing this study with the results of Gullian and Villanueva (2009) and Trushenski et al. (2012), it is suggested an increase on the ideal concentration of MS-222 and eugenol for larger cobia. However, it must also be taken in consideration

that temperatures were also different (23°C) for the smaller cobia. These results corroborate the importance of an anesthetic evaluation, not only for different species, but for different size/weight, and temperature, for a given species. For example, the optimum concentrations to anesthetize pike silverside *Menidia estor* with benzocaine at 25°C was on the range 15-18 mg L^{-1}, while at 15°C the best concentration was 12 mg L^{-1} (Ross et al. 2007). Gomes et al. (2011) anesthetized two classes (3.5 and 150 g) of silver catfish *Rhandia quelen* at different eugenol concentrations (20, 30, and 40 mg L^{-1}) and temperatures (15, 23, and 30°C), and observed that fish size and temperature affected eugenol efficacy: larger fish took longer to reach anesthesia induction, and fish acclimated to higher temperatures recovered faster for all concentrations tested.

Regarding the time for fish recovery, a common pattern noticed is the increased recovery time with increasing anesthetic concentration, and that was observed by Chambel et al. (2015) for three different species (zebrafish *Danio rerio*, guppy *Poecilia reticulate*, and discu *Symphysodon discus*) anesthetized with MS-222. However, a fourth species (green swordtail *Xiphophorus helleri*) presented a different pattern, where recovery time decreases with increasing MS-222 concentration. This was also verified for cobia anesthetized with MS-222 at 120 mg L^{-1}, as recovery time was similar for cobia at the lowest concentration (60 mg L^{-1}), but faster than those at intermediate concentrations (80 and 100 mg L^{-1}). This apparent contradictory response can be explained by the lowest time that fish stay in contact with the anesthetic, since they are anesthetized faster at higher concentrations, thus fish can expel the anesthetic in a quicker way from the bloodstream (Weber et al. 2009; Chambel et al. 2015).

Fish under stress experience a series of physiological changes, which generates primary and secondary stress responses, leading to an increase in blood cortisol and

catecholamines, with consequent increase in glucose, changes in hematological status and osmoregulation (Wendelaar Bonga 1997; Fagundes and Urbinati 2008).

Glucose is recognized as a stress indicator and hyperglycemia due to the use of anesthetics has been reported (Sladky et al. 2001; Bolasina 2006). In this study, all concentrations of both anesthetics and even the use of ethanol (eugenol vehicle) resulted in increased glucose levels after 1 h from anesthesia. Nonetheless, after 24 h, glucose levels were all similar to Pre-anesthesia. It is important to note the higher glucose level for cobia exposed to ethanol alone, while eugenol combined to ethanol prevented a higher rise in glucose, thus corroborating its anesthetic property. Trushenski et al. (2012) anesthetized larger cobia with 150 mg MS-222 L^{-1} and 60 mg eugenol L^{-1} and also found elevated levels of glucose after 1 h from exposition, which was restored to normal levels after 6 h.

Fish exposed to a stressor are subjected to increased hematocrit percentage due to a combination of erythrocyte swelling and spleen contractions and is an immediate response to stress mediated by catecholamines (Tort et al. 2002; Olsen et al. 2005). The increase of erythrocytes is a strategy to improve the ability to transport oxygen under stress conditions (Trenzado et al. 2006). In this study, hematocrit increased after exposure to 30 mg eugenol L^{-1}, however it returned to Pre-anesthesia level after 24 h. The same was observed for red pacu *Piaractus brachypomus,* which showed higher hematocrit during anesthesia with different concentrations of MS-222 and eugenol (Sladky et al. 2001). However, Brazilian codling *Urophycis brasiliensis* had hematocrit unchanged after exposure to benzocaine (Bolasina 2006).

Despite significant, the difference in osmolality levels between Pre-anesthesia and all MS-222 concentrations at 1 and 24 h was minimal. Cobia challenged to air exposure showed an increase in osmolality after 0.5 h, ranging from 368 mOsm L^{-1}

(Pre-stress) to 394 mOsm L^{-1} (Trushenski et al. 2010). Therefore, osmolality levels after anesthesia with MS-222 (362 – 368 mOsm L^{-1}) were in accordance with the literature for unstressed cobia. However, cobia exposed to ethanol showed a substantial rise in osmolality after 1 h (391 mOsm L^{-1}). Osmotic unbalance is common for stressed marine fish, increased osmolality is a typical response, due to increasing passive ion influxes and water loss, coupled to inhibited active ion exchange (Wendelaar Bonga 1997; Davis 2006). Nonetheless, osmolality for cobia exposed to ethanol was restored to control levels after 24 h.

Therefore, according to the results of the present study, MS-222 at 100 mg L^{-1} and eugenol at 30 mg L^{-1} are considered the minimum safe and effective concentrations and were the best cost-benefit anesthetics for juvenile cobia, since time to reach deep anesthesia and also to recover from it were appropriate. Other than that, the secondary stress responses were mostly restored 24 h after cobia were anesthetized.

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TABLES:

Table 1: Stages of induction and recovery from anesthesia efficacy for juvenile *Rachycentron canadum*. Modified from Park et al. (2008).

Stages	Characteristic behavior
Induction	
A1	Loss of equilibrium (sedation)
A2	Swimming stopped (deep anesthesia)
A3	Erratic opercular movement
Recovery	
R1	Normal opercular movement
R2	Swimming restarted
R3	Equilibrium recovered

Table 2: Anesthesia induction and recovery times (in seconds: mean ± SD) of juvenile cobia *Rachycentron canadum* (n=10) exposed to three concentrations of MS-222 or eugenol. Different letters indicate significant differences ($p<0.05$) among concentrations for each anesthetic at each stage of anesthesia/recovery, as indicated by one-way ANOVA and Tukey's test.

Concentration (mg L^{-1})	Induction time (s)			Recovery time (s)		
	Stage A1	Stage A2	Stage A3	Stage R1	Stage R2	Stage R3
MS-222						
60	30 ± 4^{b}	402 ± 218^{c}	837 ± 103^{c}	----	14 ± 5^{a}	88 ± 13^{a}
80	21 ± 5^{a}	85 ± 19^{b}	200 ± 46^{b}	8 ± 4^{a}	35 ± 12^{b}	135 ± 18^{b}
100	19 ± 4^{a}	47 ± 10^{a}	79 ± 24^{a}	10 ± 7^{ab}	37 ± 8^{bc}	148 ± 24^{b}
120	17 ± 2^{a}	35 ± 5^{a}	60 ± 10^{a}	14 ± 5^{b}	54 ± 18^{c}	101 ± 25^{a}
Eugenol						
20	23 ± 6^{b}	145 ± 33^{c}	197 ± 42^{d}	5 ± 6^{a}	59 ± 15^{a}	161 ± 36^{a}
30	22 ± 4^{ab}	68 ± 10^{b}	101 ± 17^{c}	22 ± 14^{a}	90 ± 24^{b}	161 ± 37^{a}
40	17 ± 3^{a}	56 ± 10^{ab}	81 ± 11^{b}	23 ± 19^{a}	81 ± 17^{ab}	187 ± 29^{a}
50	17 ± 4^{a}	46 ± 7^{a}	64 ± 5^{a}	56 ± 17^{b}	120 ± 19^{c}	171 ± 14^{a}

Table 3: Glucose (mg dL^{-1}) levels for juvenile cobia *Rachycentron canadum* (n=9) exposed to different concentrations of MS-222 and eugenol at Pre-anesthesia, 1 and 24 h after anesthesia. Different lowercase letters indicate significant differences ($p<0.05$) among different treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) from the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test.

Anesthetic	Concentration (mg L^{-1})	Time (h)		
		Pre-anesthesia	After Anesthesia	
			1	24
MS-222		58.6 ± 7.0^{A}		
	80		103.9 ± 25.9^{B}	70.7 ± 22.1^{A}
	100		106.8 ± 23.0^{B}	61.5 ± 7.7^{A}
	120		105.2 ± 20.1^{B}	60.0 ± 7.6^{A}
		64.0 ± 7.5^{A}		
Eugenol	30		99.1 ± 18.8^{xB}	65.4 ± 11.4^{A}
	40		89.1 ± 12.1^{xB}	60.8 ± 11.6^{A}
	50		105.8 ± 24.9^{xB}	61.5 ± 5.2^{A}
	0.43 mL ethanol L^{-1}		200.4 ± 59.9^{yB}	68.4 ± 13.4^{A}

Table 4: Hematocrit (%) for juvenile cobia *Rachycentron canadum* (n=9) exposed to different concentrations of MS-222 and eugenol at Pre-anesthesia 1 and 24 h after anesthesia. Different lowercase letters indicate significant differences ($p<0.05$) among different treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) from the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test.

Anesthetic	Concentration ($mg\ L^{-1}$)	Time (h)		
		Pre-anesthesia	After Anesthesia	
			1	24
MS-222		22.2±4.9		
	80		23.6±3.9	26.6±4.6
	100		26.0±6.8	27.0±7.1
	120		21.3±1.9	22.8±2.6
		$32.0±4.6^{AB}$		
Eugenol	30		$32.5±3.5^{B}$	$27.5±4.4^{A}$
	40		27.4±6.7	27.1±3.7
	50		32.2±6.1	27.2±3.5
	0.43 mL ethanol L^{-1}		30.5±3.2	29.3±6.8

Table 5: Osmolality (mOsmol kg^{-1}) levels for juvenile cobia *Rachycentron canadum* (n=9) exposed to different concentrations of MS-222 and eugenol at Pre-anesthesia, 1 and 24 h after anesthesia. Different lowercase letters indicate significant differences ($p<0.05$) among different treatments at each time interval, and different capital letters indicate significant differences ($p<0.05$) from the same treatment at different time intervals, as indicated by one-way ANOVA and Tukey's test.

Anesthetic	Concentration ($mg\ L^{-1}$)	Time (h)		
		Pre-anesthesia	After Anesthesia	
			1	24
MS-222		350.4 ± 3.7^{A}		
	80		361.7 ± 7.4^{B}	364.7 ± 5.1^{B}
	100		367.2 ± 7.0^{B}	368.4 ± 10.6^{B}
	120		363.2 ± 5.2^{B}	363.8 ± 7.1^{B}
		367.6 ± 8.7^{A}		
Eugenol	30		364.4 ± 7.9^{x}	362.0 ± 6.1
	40		370.1 ± 8.4^{x}	361.4 ± 12.7
	50		361.2 ± 9.0^{x}	366.7 ± 10.0
	0.43 mL ethanol L^{-1}		390.7 ± 12.6^{yB}	368.2 ± 7.1^{A}

DISCUSSÃO GERAL

A atenção dada ao bem-estar animal em aquicultura é relativamente recente quando comparada à criação de outros vertebrados (Brown, 2015). Assim, há a necessidade do desenvolvimento de protocolos específicos para que as técnicas de manejo sejam padronizadas para cada espécie, já que o aumento dos cuidados com a saúde animal deve imperar como questão ética, ao mesmo tempo em que reflete positivamente o aumento da produção.

Em aquicultura, o transporte de peixes é considerado uma atividade inerente e de grande importância, e já foi reportado que um dos principais problemas na produção de bijupirá é a alta mortalidade durante o transporte entre o berçário e os tanques de engorda (Liao et al., 2004). No presente trabalho, foram avaliados diferentes mecanismos para redução do estresse durante o transporte de bijupirá. Dentre esses mecanismos estão a avaliação de diferentes densidades de estocagem, utilização de tampão para manutenção do pH, redução de temperatura e salinidade e utilização dos anestésicos.

A utilização de bicarbonato de sódio (1 g L^{-1}) no transporte utilizando diferentes densidades de estocagem (15, 24 e 33 g L^{-1}) foi eficiente para a manutenção do pH acima de 7,0, permitindo o transporte de juvenis de bijupirá (30 g) numa densidade de até 24 g L^{-1} por 8 h sem mortalidade. Em 33 g L^{-1} ocorreu 18,2 % de mortalidade. O uso do bicarbonato de sódio também foi eficiente no transporte (24 h) do bacalhau *Gadus morhua* (10 g) numa densidade de 30 g L^{-1} (15°C). A concentração utilizada foi de 1 g L^{-1}, mantendo o pH acima de 7,4 durante 24 h, apesar da necessidade de uma adição extra da mesma concentração após 6 h de transporte (Treasurer, 2012).

A utilização de salinidades próximas ao ponto isosmótico auxilia na diminuição de gastos energéticos com a osmorregulação. Já a redução da temperatura diminui o metabolismo, ajudando na manutenção da qualidade da água durante o transporte (Harmon, 2009; Sampaio e Freire, 2016). No experimento em que foram utilizadas duas combinações de salinidade e temperatura (além da utilização de 1 g $NaHCO_3$ L^{-1}), o tratamento com a redução de ambos os parâmetros (S12-T19) foi o mais eficiente em relação à mortalidade e em relação à diminuição dos fatores de estresse. As mortalidades ocorreram apenas para as combinações utilizando a salinidade 30, independentemente da temperatura (S30-T19: 1,2 %; S30-T23: 7,4 %). Além disso, foi possível aumentar a densidade de transporte para 27 g L^{-1}, fato de importância econômica, já que o aumento da densidade diminui os custos da atividade (Stuart et al., 2015). Apesar de não serem observadas mortalidades para o tratamento S12-T23, este tratamento não foi recomendado pois a concentração de CO_2 na água foi muito elevada, podendo ser um fator relevante para a ocorrência de mortalidades.

A redução da temperatura para 15°C no transporte de alevinos de jundiá *Rhandia quelen* (168 g L^{-1}) por 24 h também foi eficiente, pois não ocorreu mortalidade e os parâmetros de qualidade da água foram mantidos, quando comparado ao transporte nas temperaturas 20 e 25°C (Golombieski et al., 2003). Alevinos de bijupirá (1,65 g) transportados por 24 h em duas salinidades (12 e 32 g L^{-1}) e em quatro diferentes densidades (5, 10, 15 e 20 g L^{-1}), apresentaram maior sobrevivência quando transportados na salinidade próxima ao seu ponto isosmótico, sendo que na maior densidade testada (20 g L^{-1}), ocorreram aproximadamente 35% e 90% de mortalidade nas salinidades 12 e 32 g L^{-1}, respectivamente (Stieglitz et al., 2012), corroborando com os resultados deste experimento.

Também foi observado um aumento na concentração de hemoglobina (com exceção do tratamento S30-T23) logo após o transporte, retornando aos níveis basais após 24 h. Além disso, foi observada uma diminuição no pO_2 sanguíneo após o transporte, que pode ser resultado do aumento do consumo de oxigênio devido à condição de estresse à qual os peixes foram submetidos, levando a um acúmulo de CO_2 no sangue, que também foi observado. O aumento na concentração de hemoglobina ocorreu na tentativa de manter o transporte de gases que provavelmente foi afetado pelos efeitos Root e Bohr (Wendelaar Bonga, 1997; Claiborne et al., 1999)

No transporte com o anestésico mentol, as mesmas técnicas empregadas nos experimentos anteriores foram utilizadas: adição de 1 g $NaHCO_3$ L^{-1}, redução da salinidade para 12 g L^{-1} e redução da temperatura (18,5°C). A densidade foi de 26 g L^{-1} e o tempo de transporte também foi 8 h, sem mortalidades para nenhum tratamento. Porém, um fato importante observado foi o aumento da glicemia logo após o transporte nos tratamentos em que o mentol foi adicionado, sendo 171, 184 e 258 mg dL^{-1} para os tratamentos 0, 2,5 e 5,5 mg mentol L^{-1}, respectivamente. Sabe-se que o principal efeito de um anestésico é a redução do metabolismo, contudo muitas vezes o próprio anestésico pode provocar reações de estresse (Zahl et al., 2010; Velisek et al., 2011). Assim, a exemplo do uso da benzocaína (Pedron et al., 2016) , a adição de mentol não melhorou as condições de transporte para bijupirá. Sepulchro et al. (2016) utilizaram mentol (0, 3,7 e 7,4 mg L^{-1}) para o transporte de robalo-peva *Centropomus parallelus* (1,6 g/ densidade 16 g L^{-1}/ 26,7°C) em três diferentes salinidades (0, 17 e 36 g L^{-1}), e também não recomendaram o uso do anestésico. Em contrapartida, Navarro et al. (2016) transportaram juvenis de tilápia *Oreochromis niloticus* por 10 h com duas diferentes concentrações de mentol (75 e 100 mg L^{-1}), e observaram redução do cortisol e manutenção dos níveis de glicemia para a concentração de 75 mg mentol L^{-1} quando

comparado ao tratamento sem anestésico, sendo 75 mg L^{-1} a concentração indicada de mentol para o seu transporte.

Assim como no transporte com diferentes salinidades e temperaturas, durante o transporte com a utilização de mentol ocorreu uma redução de O_2 e um aumento do CO_2 no sangue dos peixes, apesar de na água as concentrações de O_2 e CO_2 estarem dentro de níveis considerados normais (13 mg O_2 L^{-1} e 28 g CO_2 L^{-1}). Na mesma tentativa de melhorar o transporte de gases, os peixes também aumentaram seus níveis de hemoglobina e hematócrito (Wendelaar Bonga, 1997; Claiborne at al., 2002).

Tricaína metanosulfato (MS-222), eugenol, benzocaína, 2-fenoxietanol e metomidato são os anestésicos mais utilizados em aquicultura (Husen e Sharma, 2014). Outros anestésicos advindos de extratos vegetais também vêm sendo testados, como por exemplo, o mentol, os extratos de *Spilanthes acmella*, *Aloysia gratissima* e *Ocimum gratissimum* (Benovit et al., 2012; Kasai et al., 2014; Barbas et al., 2016).

Neste trabalho, foram testados os anestésicos MS-222, eugenol e mentol para serem utilizados durante práticas de manejo como, por exemplo, biometria e coleta de sangue. As concentrações utilizadas foram: 60, 80, 100 e 120 mg MS-222 L^{-1}, 20, 30, 40 e 50 mg eugenol L^{-1} e 35, 45 e 55 mg mentol L^{-1}. De acordo com Marking e Meyer (1985), dentre as várias qualidades que um anestésico necessita para ser considerado ideal, está em ser capaz de anestesiar o peixe em até 3 min. e que ele se recupere em até 5 min. Este resultado pôde ser observado para as concentrações 100 e 120 mg MS-222 L^{-1}, para as concentrações 30, 40 e 50 de eugenol e para a concentração 55 mg mentol L^{-1}. Assim, considerando o argumento de Marking e Meyer (1985) e a menor relação custo-benefício, as concentrações mais eficientes para juvenis de bijupirá (≈ 50 g/ 26,5°C) foram 100 mg MS-222 L^{-1}, 30 mg eugenol L^{-1} e 55 mg mentol L^{-1}. Se formos

eleger um anestésico entre os três avaliados, o eugenol foi o mais eficiente em termos de custo-benefício.

Gullian e Villanueva (2009) anestesiaram juvenis de bijupirá de menor tamanho (5-14 g/ 23°C), e recomendaram o uso de 60 mg MS-222 L^{-1} e 20 mg eugenol L^{-1}. Já Trushenski et al. (2012) recomendaram o uso de 150 mg MS-222 L^{-1} e 60 mg eugenol L^{-1} para bijupirás de 300 g (27°C). Estas diferentes concentrações estão de acordo com o fato de que a eficiência de um anestésico pode variar de acordo com fatores biológicos como o tamanho do peixe, mesmo o indivíduo sendo da mesma espécie e fatores ambientais como a temperatura (Ross e Ross, 2008).

Apesar de todos os anestésicos terem induzido anestesia, não foram capazes de conter a elevação dos valores de glicemia após exposição, indo de acordo com a idéia de que o anestésico em si pode causar resposta de estresse (Zahl et al., 2010; Velisek et al., 2011). No trabalho de Trushenski et al. (2012) também com bijupirá, observou-se o mesmo padrão de aumento na glicemia após 0,5, 1 e 2 h da exposição e além disso, não houve supressão do cortisol. Palic et al. (2006) testaram várias concentrações de MS-222 (25 – 200 mg L^{-1}) e eugenol (10 – 80 mg L^{-1}) para *Pimephales promelas* (3 g) e encontraram como concentração ideal 75 e 30 mg L^{-1} para MS-222 e eugenol, respectivamente, sendo que após submissão a teste de estresse, apenas o eugenol foi eficiente em reduzir os níveis de cortisol. Já Simões e Gomes (2009) anestesiaram juvenis de tilápia do Nilo (14 g) e concluíram que as concentrações entre 150 e 200 mg L^{-1} de mentol foram eficientes para o manejo, apesar de também provocarem o aumento da glicemia.

Assim, os resultados do presente estudo indicam que a adição de 1 g $NaHCO_3$ L^{-1}, a redução da salinidade da água para 12 g L^{-1} e diminuição da temperatura para 19°C, são técnicas eficazes para aumentar a densidade de estocagem (27 g L^{-1}) e reduzir a

resposta de estresse durante o transporte de juvenis de bijupirá (≈ 30 g). Já a adição de mentol não foi eficiente durante o transporte, porém foi capaz de induzir anestesia na concentração de 55 mg mentol L^{-1}, assim como MS-222 na concentração de 100 mg L^{-1} e eugenol 30 mg L^{-1}.

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CONCLUSÕES

- A utilização de 1 g $NaHCO_3$ L^{-1} foi eficiente para manter o pH levemente alcalino durante o transporte de juvenis de bijupirá, não ocorrendo mortalidades até a densidade de 24 g L^{-1}.
- A diminuição da salinidade da água para níveis próximos ao seu ponto isosmótico (≈ 12 g L^{-1}) e a redução da temperatura (≈ 19 °C) são técnicas eficazes para aumentar a densidade de estocagem (27 g L^{-1}) e reduzir a resposta de estresse durante o transporte de juvenis de bijupirá.
- A concentração de 55 mg mentol L^{-1} foi eficiente para induzir a anestesia em juvenis de bijupirá, porém o anestésico mentol não é recomendado para ser utilizado durante o transporte, já que não apresentou efeitos benéficos como melhora na qualidade da água ou redução do estresse.
- Os anestésicos MS-222 e eugenol foram eficientes em induzir anestesia e com melhor custo-benefício nas concentrações 100 e 30 mg L^{-1}, respectivamente.