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## Physiological and hematological responses of Nile tilapia (*Oreochromis Niloticus*) to different anesthetics during simulated transport conditions

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**ABSTRACT.** Minimization of stress during the transportation of live fish is essential in maintaining the welfare and performance of the animals. In order to test the hypothesis that stress during transport of fingerlings of Nile tilapia (*Oreochromis niloticus*) can be reduced with the aid of the anesthetics menthol, eugenol or benzocaine, we have assessed the effects of these agents at various concentrations on the physiological parameters and survival rates of fish subjected to conditions simulating those normally used in transportation. Fingerlings (N = 1200) were fasted for 24 hours and distributed in 20 L polyethylene bags (N = 50 per bag) containing 5 L of water and an anesthetic at the appropriate concentration. Fingerlings treated with menthol at 75 mg L<sup>-1</sup>, or eugenol or benzocaine at 20 mg L<sup>-1</sup>, maintained levels of plasma cortisol and glucose that were lower than those of the stressed but untreated controls and within the physiological limits of the baseline values for this species. Under these conditions, the survival rate was 100%, suggesting that stress was substantially reduced despite dense consignment. Treatments involving higher doses of the studied agents induced significant anesthetic toxicity.

**Keywords:** menthol, benzocaine, eugenol, fish.

### Resposta fisiológica de tilápia do Nilo (*Oreochromis niloticus*), sobre diferentes anestésicos em situação de transporte

**RESUMO.** Minimização de estresse durante o transporte de peixes vivos é essencial para manter o bem-estar e o desempenho dos animais. A fim de testar a hipótese de que o estresse durante o transporte de alevinos de tilápia do Nilo (*Oreochromis niloticus*) pode ser reduzido com o auxílio dos anestésicos mentol, eugenol ou benzocaína, que avaliaram os efeitos destes agentes em várias concentrações dos parâmetros fisiológicos e taxas de sobrevivência dos peixes submetidos a condições que simulam os normalmente utilizados no transporte. Alevinos (n = 1200) foram mantidos em jejum durante 24 h e distribuídos em sacos de 20 L de polietileno (N = 50 por sacos) contendo 5 L de água e um anestésico na concentração adequada. Alevinos tratados com mentol a 75 mg L<sup>-1</sup>, ou eugenol ou benzocaína a 20 mg L<sup>-1</sup>, mantiveram níveis de cortisol no plasma e de glucose que foram menores do que os dos controles não tratados e estressados, mas dentro dos limites fisiológicos dos valores da linha de base para esta espécie. Sob estas condições, a taxa de sobrevivência foi de 100%, o que sugere que o estresse foi substancialmente reduzido, apesar da remessa densa. Os tratamentos que envolvem doses mais elevadas dos agentes estudados induzindo uma toxicidade anestésica mais significativa.

**Palavras-chave:** mentol, bezocaína, eugenol, peixe.

### Introduction

The application of an anesthetic is often necessary in fish farming in order to immobilize the animals during handling, spawning and transport, and for operations such as gathering biometric information, carrying out pathological analysis and performing surgery. These routine procedures can

generate various stressors that have the potential to affect the performance and welfare of the fish (Takahashi, Abreu, Biller, & Urbinati, 2006; Vidal et al., 2008; Weber, Peleteiro, Martín, & Aldegunde, 2009; Delbon & Paiva, 2012). The implementation of methods that would allow such activities to be accomplished with minimal interference to vital and

physiological functions is, therefore, crucial in maintaining the welfare of the fish throughout the production process (Ueta, Suzuki, Sugimoto, Uchida, & Mashimo, 2007; Brandão, Gomes, Crescêncio, & Carvalho, 2008; Adamante, Nuñez, Barcellos, Soso, & Finco, 2008).

The physiological responses of fish to stressors are generally classified as primary and secondary (Gonçalves, Santos, Fernandes, & Takahashi, 2008; Zahl, Kiessling, Samuelsen, & Hansen, 2009), and each of these is associated with alterations in specific hematological or biochemical parameters that may be employed as stress indicators (Barbosa, Moraes, & Inoue, 2007; Morgan & Iwama, 1997). Thus, increased blood cortisol concentration is an important primary response whereas elevation of glycemia and hematological alterations are typical of secondary responses (McDonald & Milligan, 1997).

According to Luz, Costa, Silva, and Rosa (2013) the successful transportation of live fish involves consigning the maximum number of animals in the minimum volume of water without causing mortality, deterioration of water quality (as determined by temperature, oxygen content and pH) or stress. In order to fulfill these criteria while, at the same time, reducing costs and avoiding losses during long periods of transportation, methods have been developed to minimize stress and reduce mortality under dense consignment (Vidal, Albinati, Albinati, & Mécêdo, 2006; Park et al., 2008). In this context, anesthetic drugs such as menthol, eugenol and benzocaine have been frequently used to reduce the stress caused by agitation of fish during transport (Park et al., 2008), and the positive effects of adding sodium chloride to the water have been reported by several researchers (Gomes et al., 2006).

Various approaches are available for the induction of anesthesia in fish prior to transportation, and the selection of a suitable procedure depends essentially on the behavioral characteristics of the animal. Determination of the time required for an animal to reach the level of anesthesia required is very important in fish management, since the duration of each stage of anesthesia varies according to the species, the drug, the method of induction and the biological and environmental conditions of the animals (Ross & Ross, 2008; Okamura et al., 2010).

The efficiency of an anesthetic drug is determined by a number of factors, including ease of use with low risk to animals and man, high stability in solution, low toxicity, absence of allergic reactions, absence of persistent effects on behavior

following successive anesthesia procedures, rapid induction and recovery times (around 3 and 5 min, respectively), good penetration to the sites of action, rapid biotransformation in the body and high biocompatibility (Ross & Ross, 2008).

Moreover, the choice of an anesthetic depends on the application for which it is required, since each drug exhibits a different capacity for reducing metabolic rate, thereby affecting the potential production of ammonia, the consumption of oxygen, and the visual stimulus and activity of the animal (Kaiser et al., 2006; Mello et al., 2012). Clove oil, for example, has been used successfully to anesthetize fish during short-term handling. This oil, which contains between 70 and 95% of the potent natural anesthetic eugenol (Kaiser et al., 2006; Rotili et al., 2012), is obtained commercially by distillation of leaves, stems and flowers of *Syzygium aromaticum* (L.) Merr. & L. M. Perry (Myrtaceae). The synthetic drug benzocaine (ethyl 4-aminobenzoate) is a white crystalline material that is often used for anesthetizing fish by immersion prior to handling (Ross & Ross, 2008). Benzocaine is similar to the topical anesthetic tricaine methanesulfonate (MS-222) commonly employed in the sedation, immobilization and anesthesia of poikilothermic animals. Menthol is a monoterpenoid with antispasmodic, anti-inflammatory, antiviral and anti-ulcer properties, as well as irritant activity, and widely used in pharmaceuticals (Gimbo, Saita, Gonçalves, & Takahashi, 2008; Simões & Gomes, 2009).

Although there are a number of reports on the application of these anesthetics in fish transport, little is known about the effects of these drugs on Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) maintained under dense consignment. In order to examine the hypothesis that stress during transport of Nile tilapia fingerlings can be reduced using appropriate doses of anesthetics, we assessed the effects of various concentrations of menthol, eugenol and benzocaine on the time interval for anesthesia induction, changes in biochemical and hematological parameters, and the survival rates of fingerlings by simulating the conditions normally used for transport.

## Material and methods

The study was approved by the Ethical Committee of the Universidade de Brasília (protocol CEUA/UnB 51694/2011). Experiments were carried out in the laboratories of the Secretaria

de Agricultura e Desenvolvimento Rural (Seagri) in Brasília, Distrito Federal State, Brazil.

The animal population comprised 1050 fingerlings of Nile tilapia with average weight of  $5.0 \pm 2.1$  g. The experiment was of randomized design and involved subjecting fish to stress under seven treatment conditions (with three repetitions of each) comprising application of: no anesthetic (T1), menthol at 75 (T2) or 100 mg L<sup>-1</sup> (T3), eugenol at 20 (T4) or 30 mg L<sup>-1</sup> (T5), or benzocaine at 20 (T6) or 40 mg L<sup>-1</sup> (T7). Fish were fasted for 24 hours (a procedure that is generally recommended in preparing fish for transportation) and subsequently transferred to seven clear 20 L polyethylene bags (50 fingerlings per bag), each containing 5 L of water and the appropriate concentration of an anesthetic as described above. The bags were maintained at room temperature for 3.5 hours, which is the average duration of transport of tilapia.

The effects of the anesthetics on the behavior of the fingerlings were assessed according to the guidelines proposed by Wood et al. (2002) and Ross and Ross (2008). At the end of the experimental period, blood samples of 10 fish per treatment (1 mL) were collected by cardiac puncture using syringes containing anticoagulant (10% ethylenediaminetetraacetic acid). Hematocrit was determined the packed cell volume (%) following centrifugation of blood in Microhematocrit tubes (Mourent, Good, & Bell, 2005). Plasma was obtained by centrifugation of total blood for 10 min at 5,000 rpm in an Eppendorf (Hamburg, Germany) model 5415C benchtop centrifuge, followed by separation using a digital pipette. Samples of plasma were transferred to labeled Eppendorf tubes and stored in the freezer at -20°C until required for analysis. Glucose was quantified by treatment of 10 fish using the Accu-Chek Softclick digital monitor (Roche Diagnostics Corp., Indianapolis, IN, USA). Total protein concentration was assessed using an Atago (Tokyo, Japan) model SPR-N clinical refractometer. Plasma cortisol was estimated with the aid of a human enzyme-linked immunosorbent assay (Elisa) cortisol 96T kit (Abnova Corporation, Jhongli City, Taiwan).

Data normality was tested according to Kolmogorov-Smirnov (Massey Junior, 1951). Mean values of parameters determined during the different treatments were compared using the Duncan test with the statistical significance set at 5% probability. All statistical analyses were performed with the aid of SAS® software (Statistical Analysis System; Institute for Advanced Analytics, Raleigh, United States).

## Results

The aqueous medium into which the fingerlings were transferred at the start of the experiment presented a pH of  $7.0 \pm 0.51$  with a dissolved oxygen content of  $6.64 \pm 0.36$  mg L<sup>-1</sup> and a temperature of  $21.74 \pm 0.43$ °C. Small, but statistically insignificant, changes were recorded during the experimental period such that the final pH was 5.5, the dissolved oxygen content was  $5.90 \pm 0.1$  mg L<sup>-1</sup> and the temperature was  $21.33 \pm 0.03$ °C. Regarding the intervals for the induction of anesthesia, those for treatments T3, T5 and T7 were significantly shorter ( $p < 0.05$ ) in comparison with T2, T4 and T6 (Table 1). The most extended induction period of 221 s, recorded for T4, was 15.8-times longer than the shortest induction time exhibited by T3.

At the end of the experimental period, the levels of glucose in fingerlings exposed to T3, T5 and T7 were significantly ( $p < 0.05$ ) greater ( $\leq 3$ -fold) than those of the control T1 and the other treatments (Table 1). However, only fingerlings exposed to T3 and T7 exhibited levels of cortisol that were significantly ( $p < 0.05$ ) elevated ( $\leq 1.7$ -fold) in comparison with the control T1 and the other treatments. The hematocrit values of fingerlings exposed to T3 and T5 were significantly ( $p < 0.05$ ) lower than those of the control T1, but none of the treatments influenced the total protein levels. The survival rates of tilapia fingerlings were significantly reduced following exposure to T3 and T5 for 3.5 hours, with 42.5% mortality recorded for T5.

**Table 1.** Mean values ( $\pm$  standard error) of the response variables to anesthesia induced by different substances.

Treatment	Interval to anesthesia (s)	Plasma glucose (mg dL <sup>-1</sup> )	Hematocrit (%)	Plasma cortisol (ng mL <sup>-1</sup> )	Total plasma protein (g dL <sup>-1</sup> )	Survival rate (%)
T1 - Control	–	$87.38 \pm 19.25^a$	$23.20 \pm 2.0^a$	$6.74 \pm 5.89^b$	$4.28 \pm 1.0^a$	$100.00 \pm 0.0^a$
T2 - Menthol 75 mg L <sup>-1</sup>	$180.33 \pm 158.17^a$	$65.00 \pm 5.5^a$	$22.75 \pm 2.5^a$	$1.9 \pm 1.38^c$	$3.80 \pm 1.5^a$	$100.00 \pm 0.0^a$
T3 - Menthol 100 mg L <sup>-1</sup>	$14.00 \pm 14.42^b$	$265.67 \pm 109.94^b$	$16.50 \pm 2.3^b$	$8.1 \pm 0.97^a$	$4.00 \pm 1.0^a$	$87.50 \pm 10.0^b$
T4 - Eugenol 20 mg L <sup>-1</sup>	$221.00 \pm 241.12^a$	$49.67 \pm 21.2^a$	$22.25 \pm 1.6^a$	$3.9 \pm 2.70^{bc}$	$3.85 \pm 0.8^a$	$100.00 \pm 0.0^a$
T5 - Eugenol 30 mg L <sup>-1</sup>	$44.67 \pm 65.30^b$	$221.67 \pm 45.42^b$	$13.50 \pm 2.3^b$	$6.6 \pm 4.59^b$	$4.20 \pm 0.5^a$	$57.50 \pm 3.5^c$
T6 - Benzocaine 20 mg L <sup>-1</sup>	$141.67 \pm 148.05^a$	$50.67 \pm 5.43^a$	$21.50 \pm 1.4^a$	$2.8 \pm 2.44^{bc}$	$4.95 \pm 0.4^a$	$100.00 \pm 0.0^a$
T7 - Benzocaine 40 mg L <sup>-1</sup>	$32.00 \pm 27.0^b$	$100.00 \pm 14.36^b$	$18.00 \pm 1.3^a$	$11.7 \pm 3.87^a$	$4.60 \pm 0.3^a$	$100.00 \pm 0.0^a$

In each column, mean values bearing dissimilar lower case superscript letters indicate significant between-treatment differences according to Duncan test ( $p \leq 0.05$ ).

## Discussion

Levels of plasma glucose and cortisol have frequently been employed as indicators of stress in fish (Morgan & Iwama, 1997). It is well documented that stress promotes an increase in the synthesis of the glucocorticoid hormone cortisol (Martínez-Porchas, Martínez-Córdova, & Ramos-Enriquez, 2009) and the activation of glycogenolysis and glyconeogenesis, the two pathways involved in the fight-or-flight response and in the regulation of glucose levels in the blood. Thus, increasing plasma glucose concentration represents an immediate response to the elevation of metabolic and respiratory rates in muscle cells (Martínez-Porchas et al., 2009).

In the present study, fingerlings that had been exposed to high concentrations of menthol and eugenol (treatments T3 and T5) presented glucose levels that were increased significantly in comparison with the control (T1) indicating a compensatory physiological response to augmented energy demand. In previous studies, Oliveira, Carmo, and Oliveira (2009) demonstrated that exposure of Nile tilapia to 40 mg mL<sup>-1</sup> of benzocaine led to an increase in plasma glucose, while Sladky, Swanson, Stoskopf, Loomis, and Lewbart (2001) reported the augmentation of plasma glucose in Amazonian pacu (*Piaractus brachipomus* Cuvier, 1817) anesthetized with MS-222 or clove oil.

In fingerlings that had been submitted to T3 and T5, the increased levels of glucose were accompanied by reductions in hematocrit, a parameter that is associated with stress-induced secondary responses (McDonald & Milligan, 1997). In this context, it is worth noting that hematocrit is not only an indicator of chronic stress, as suggested by previous studies (Gomes et al., 2006), but also of acute stress. The significantly reduced survival rates, together with the alterations in biochemical and hematological parameters recorded in fingerlings that had been exposed to menthol at 100 (T3) and eugenol at 30 mg L<sup>-1</sup> (T5), confirm that these conditions were most unfavorable for the transport of Nile tilapia. It is likely that the level of toxicity associated with high concentrations of menthol and eugenol promoted the collapse of vital functions (depression of the central nervous system with respiratory and cardiac failure) in the anesthetized fish. Interestingly, although treatment with benzocaine at 40 mg L<sup>-1</sup> (T7) produced significant increases in plasma cortisol and glucose levels, the observed reduction in hematocrit was not statistically significant and fingerling survival rate was 100%.

Nile tilapia fingerlings exposed to menthol at 75 (T2), eugenol at 20 (T4) or benzocaine at 20 mg L<sup>-1</sup> (T6) maintained levels of plasma cortisol and glucose that were lower than those of the stressed but untreated controls (T1) and within the physiological limits of the baseline values for this species. It would appear, therefore, that these treatments were able to control stress in fingerlings under dense consignment while minimizing mortality caused by anesthetic toxicity. Although the intervals to anesthesia were significantly longer in T2, T4 and T6 compared with the other treatments, these would not impact on management efficiency since a delay of less than 5 min is inconsequential considering the total time of simulated transport (3.5 hours). In previous studies, Barham, Caiger, and Visser (1979) recommended concentrations of benzocaine in the range of 25 to 100 mg L<sup>-1</sup> for anesthesia induction in largescale mullet (*Liza macrolepis* Smith, 1846). Additionally, Mattson and Ripple (1989) reported that 40 mg L<sup>-1</sup> of benzocaine induced anesthesia very rapidly in Atlantic cod (*Gadus morhua* – Linnaeus, 1758), whereas Gomes et al. (2001) established that 100-150 mg L<sup>-1</sup> of benzocaine was suitable for tambaqui (*Colossoma macropomum* G. Cuvier, 1818).

## Conclusion

In conclusion, the results obtained in this study provide evidence in support of the hypothesis that stress during transport of Nile tilapia fingerlings can be reduced using the anesthetics menthol, eugenol or benzocaine, although care must be taken with regard to the dosage employed in order to ensure the safety and welfare of the animals. While many fish species can tolerate relatively high concentrations of these anesthetics, Nile tilapia appear to be somewhat susceptible to their toxic effects, and applied doses must be ≤ 75 mg L<sup>-1</sup> for menthol and ≤ 20 mg L<sup>-1</sup> for eugenol and benzocaine. The information provided herein can direct new research to improve handling and transport of Nile tilapia and, therefore, contribute to good farm management practices and the production of healthy fingerlings for aquaculture.

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