Stress responses to handling in Nile tilapia (*Oreochromis niloticus* Linnaeus): assessment of eugenol as an alternative anesthetic

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ABSTRACT. Eugenol, the main component of clove oil, has been proposed as an alternative fish anesthetic with no apparent toxic effects to people and environment. In addition, anesthesia may reduce stress and risk of trauma to fish during handling. Therefore, the use of anesthetics may reduce fish mortality. However, studies are required on short-term exposures to eugenol to assure the target animal safety of this product. The present work reports evaluation of biochemical responses of Nile tilapia to handling with concurrent two environmental concentrations of eugenol. Based on the results of this study, eugenol appears to be a safe anesthetic for use in this species.

Key words: anesthetic, eugenol, handling, stress, tilapia.

RESUMO. Respostas metabólicas da tilápia do Nilo (*Oreochromis niloticus*) submetida ao manuseio e ao anestésico eugenol. O eugenol é o principal componente do óleo de cravo, sendo proposto como anestésico alternativo para peixes, pois aparentemente não apresenta características tóxicas aos trabalhadores e ao meio ambiente. Ainda, o uso de anestésicos durante o manejo de peixes pode reduzir o estresse e riscos de acidentes pela movimentação excessiva dos animais. Portanto, a anestesia em peixes pode evitar mortalidade durante o manejo. Entretanto, ainda são necessários estudos sobre os efeitos do eugenol em peixes, o que pode assegurar a sua viabilidade de uso. O presente trabalho avaliou as respostas bioquímicas da tilápia do Nilo submetida ao manuseio e a duas concentrações de eugenol. Os resultados indicam esse produto como seguro para uso nessa espécie.

Palavras-chave: anestésico, eugenol, estresse, tilápia.

Introduction

The benefits of reducing the handling stress through the use of anesthetics are controversial (Walsh and Pease, 2002). Studies on this topic report significant increases of plasma cortisol and glucose especially when fish are exposed to tricaine methanosulfonate MS 222 (Roubach *et al.,* 2001), benzocaine (Gomes *et al.,* 2001), clove oil (Cho and Heath, 2000), 2-phenoxyethanol (Tort *et al.,* 2002), and metomidate (Iversen *et al.,* 2003). Nonetheless, anesthetics are usually necessary in fish biology procedures to avoid handling trauma. Aquaculturists need anesthetics to immobilize fish for field practices such as biometry, injections, and tagging. Handling trauma may have serious consequences as bacteria and fungi infections, and subsequent fish mortality (Durville and Collet, 2001). Therefore, whenever possible, the use of anesthetics is recommended for safe handling of fish. Furthermore, ethics issues in animal use require the anesthetics during field and lab procedures to avoid or minimize fish suffering. However, some factors must be considered in the selection and use of anesthetics.

The market availability and cost play a pivotal role in the choice of an anesthetic. In some countries as United States, the US Food and Drug Administration currently only label as fish anesthetic tricaine methanosulfonate (MS 222), followed by a minimum 21-days withdrawal period (Roubach *et al.,* 2001). However, MS 222 is expensive and frequently unavailable in many countries due to several international restrictive rules regarding importation of this chemical (Roubach and Gomes, 2001).

Eugenol, the main component of clove oil, has been reported as an alternative fish anesthetic, currently without any evident toxic effects to fish, humans or environment (Kildea *et al.,* 2004). Anesthetic and sedative effects of eugenol are quickly observed in fish

exposed via baths, and positive results, including the decrease of some physiological stress responses during hauling and transport, are observed (Inoue *et al.,* 2003; 2005). Eugenol has been used for many years for purposes other than aquaculture such as dentistry as a component of temporary dental fillings. In the past, eugenol in the clove oil form was used as part of homemade analgesics for toothache. Currently, food industries use this natural product for flavor purposes.

Anesthesia in fish is a result of depression of the functions at the nervous system, but the exact mechanisms involved are still unclear (Ross and Ross, 1999). Fish anesthesia is classified in relation to the loss of equilibrium and the ability of a fish to regain the upright position (Iwama and Ackerman, 1994; Woody *et al.,* 2001). Basically, four anesthesia stages are considered. Stage 3, characterized by the complete loss of equilibrium and inability to recover the upright position (Woody *et al.*, 2001), is the most common used in fish farming. Eugenol appears to depress the skeletal muscle by a calcium potential unbalance of the nervous system receptors (Lahlou *et al.,* 2004). However, additional information is necessary to understand the eugenol mechanisms of action as well as the consequences of its use. Ideal concentrations, any associated effects, safety to humans and withdrawal periods need to be more investigated. Currently, the lack of information regarding anesthetics in different fish species tends to mislead professionals in their assumptions that stress will be reduced (Iwama and Ackerman, 1994).

Nile tilapia (*Oreochromis niloticus*) is the most cultivated fish in Brazil as well in several other countries in the world. This species has excellent growth and feed conversion rates (Popma and Lovinsh, 1995). The presence of spines and sharp bony fins may result in risk of injury to the fisheries workers. Use of anesthetics may reduce this risk.

In the present work, responses of Nile tilapia to handling and short-term exposures to eugenol were evaluated. The applicability and convenience for such anesthetic are discussed. Handling and handling plus anesthesia stages were compared. Finally, a protocol using a metabolic approach was performed to estimate the responses of Nile tilapia to handling, and to investigate the possible stress-reducing effect of eugenol.

Material and methods

Eugenol used in the trials was a pharmaceutical grade product (SW 1000 mg mL^{-1}), purchased in a local dentistry drugstore. It was diluted in ethanol (one part of eugenol to 20 parts of ethanol) to avoid undesirable hydrophobic characteristics. The final eugenol concentration in the alcoholic solution was 50 mg mL^{-1}.

One hundred twenty fish (58.6±23.8 g, and 14.6±1.7 cm) were randomly and equally distributed in twelve 250-L tanks, supplied in a closed re-circulating system with aerated water, heated to 25ºC, and filtrated through sand. Water was free of organic matter, ammonia and nitrate as the system was partially replaced everyday (about 15%). The parameters daily monitored were pH 6.8±0.2, dissolved oxygen 5.66±0.07 mL^{-1}, and water conductivity 74.3±4.8 μS cm^{-1}. Fish were fed with commercial pellets (30% crude protein) twice a day for 15 days. Twenty-four hours prior to the experiment the feeding was discontinued. The experimental design included 4 treatments with 3 replications each (sub-groups) to perform handling stress plus the anesthetic effect of eugenol. The experimental groups were: T1) control, T2) handling, T3) handling plus anesthesia, T4) handling plus deep anesthesia. The handling-group fish (T2) were transferred to glass aquaria (26 x 17 x 14 cm) with 2 L of water and aeration for 10 min. After that, fish were removed to 20-L buckets with water and aeration for additional 20min, and then returned to the 250-L tanks. The fish plus anesthesia group (T3) was subjected to the same treatment of handling (T2), except by 20 mg L^{-1} of eugenol which was added to the glass aquaria. The handling plus deep anesthesia group (T4) was handled as groups T2 and T3, but the eugenol concentration added to the glass aquaria was 80 mg L^{-1}. Following the return of fish to the 250-L tanks, five fish were immediately sampled. The remaining 5 fish were allowed to recover for 6 hours and then they were sampled. Fish from the control group (T1-not subjected to neither handling nor eugenol) were only quickly sampled with a dip-net at the time 0 and 6 hours followed the same procedure for T2, T3 and T4. All the procedures were carried out simultaneously.

After sampled, the fish were killed by cervical section (0 and 6h after stressors). Blood was collected in heparinzed syringes from the caudal vein; samples of liver and white muscle were excised and immediately transferred to liquid nitrogen for analyses of glycogen (Bidinotto *et al.,* 1997). Blood aliquots were centrifuged at 14,400 x g for 3min at 4ºC and the plasma was separated for determination of: glucose (Trinder, 1969), total protein (Kruger, 1994), sodium and potassium (flame photometry), chloride (Schales and Schales, 1941), lactate (Harrower and Brown, 1972), and total ammonia (Gentzkow and Masen, 1942).

The data were analyzed through the software Graph Pad Instat for ANOVA. Tukey's test for means comparison was also carried out through the Graph Pad Instat. The significance selected level was $P<0.05$. All the values are expressed as mean ± standard error of mean (s.e.m.).

Results and discussion

Plasma glucose concentrations were significantly elevated ($P<0.05$) after handling. All fish exposed to eugenol (T3 and T4) had the same biochemical responses compared t]o the handled group (T2). Glucose concentration returned to basal levels 6 hours after recovery in all groups (Figure 1). Liver glycogen concentration was the same (610±150 μmol g^{-1}) in all experimental conditions. White muscle glycogen concentration decreased in T2 and T3. However, deep anesthesia (T4) prevented some consumption of muscular glycogen. The content of white muscle glycogen after 6 hours of recovery decreased significantly ($P<0.05$) in all treatments (Figure 2). Plasma levels of lactate were increased after handling (Figure 3). Lower concentrations of lactate were observed in handled fish exposed to eugenol for light anesthesia (T3). However, fish submitted to handling plus deep anesthesia (T4) showed the highest plasma lactate concentrations. Total plasma ammonia concentrations were equally elevated after all handling treatments, but the basal levels were recovered 6 hours after the stress (Figure 4). Plasma ions concentration (Na^+ = 146.1±7 mEq L^{-1}, Cl^- = 201.6±14 mEq L^{-1}, K^+ = 6.1±2 mEq L^{-1}) and plasma protein content (68.6±9 mg mL^{-1}) were not affected by the treatments.

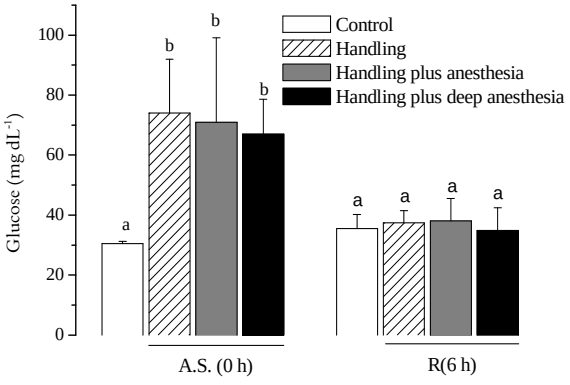

Figure 1. Plasma glucose concentration (mg dL^{-1}; mean ± s.e.m.) of *Oreochromis niloticus* in response to handling and short-term exposures to eugenol. (A.S.) fish sampled immediately after stressors and (R) 6-h-recovery. Distinct letters means significant difference at $P<0.05$.

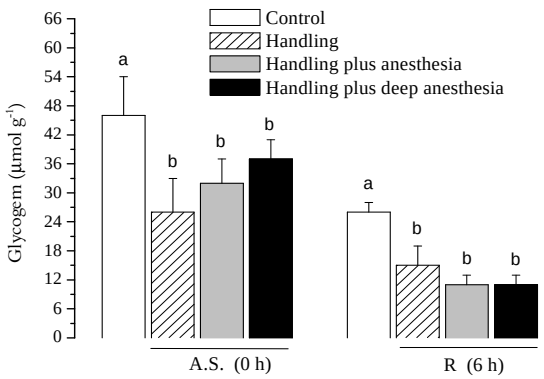

Figure 2. Muscular glycogen (mean ± s.e.m.) of *Oreochromis niloticus* in response to handling and handling plus short-term exposures to eugenol. (A.S.) fish sampled immediately after stressors and (R) 6-h-recovery. Distinct letters means significant difference at $P<0.05$.

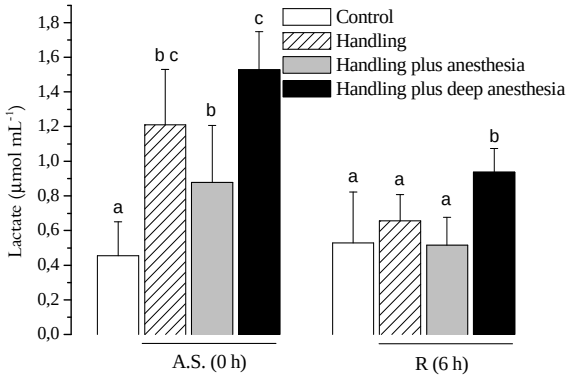

Figure 3. Plasma lactate concentration (mean ± s.e.m.) of *Oreochromis niloticus* in response to handling and handling plus short-term exposures to eugenol. (A.S.) fish sampled immediately after stressors, and (R) 6-h-recovery. Distinct letters means significant difference at $P<0.05$.

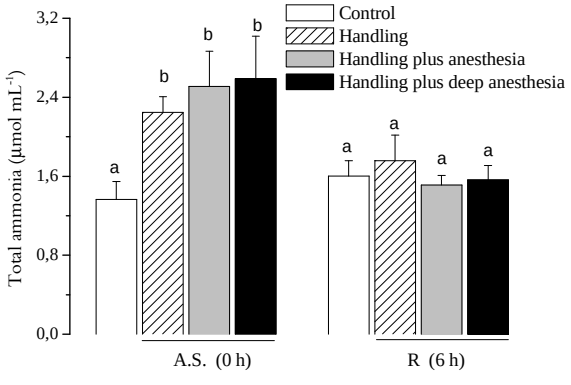

Figure 4. Plasma ammonia concentration (mean ± s.e.m.) of *Oreochromis niloticus* in response to handling and short-term exposures to eugenol. (A.S.) fish sampled immediately after stressors and (R) 6-h-recovery. Distinct letters means significant difference at $P<0.05$.

The use of eugenol has been reported as an effective anesthetic for some fish species (Munday and Wilson, 1997; Sladky *et al.,* 2001; Inoue *et al.,* 2003). This product is cheap, available in drugstores and apparently non-toxic (Woody *et al.,* 2001). Furthermore, antiseptic characteristics have been reported as well as a pleasant odor (Soto and Burhanuddin, 1995). Eugenol is completely eliminated from fish tissues 48h after exposures (Kildea *et al.,* 2004). Nile tilapia was satisfactorily anesthetized, and it was handled easily after anesthesia. This assures its safe handling after eugenol exposition preventing any risk to handlers, fish biologists and fish culturists. However, fish overexposure to eugenol must be avoided (Durville and Collet, 2001).

Changes of plasma glucose concentrations may reflect a stressing condition in fish. This parameter is widely used to gauge a stress response in the field (Hattingh, 1976). The treatment handling of the Nile tilapia resulted in a significant plasma glucose rise. Curiously, the same response was also observed in fish exposed to eugenol. Eugenol was expected to reduce some of the effects of handling. Therefore, it was expected that the anesthetized fish would have plasma glucose concentrations near to those of the control group. Considering the fact that fish experienced anesthetic effects within few seconds of eugenol exposures, one can assume that some stress was imposed to them during the short time prior to the pharmacological effects. The most reasonable explanation would be that capture of fish through a dip net plus the transfer to the experimental aquarium was enough to induce catecholamine release and subsequent glucose rise. This is a classic response of fish to cope with stress. The increase of plasma glucose observed in this work does not guarantee that eugenol exposures minimize handling effects. However, it is also not possible to state that additional stress is caused by the anesthetic. Alternatively, eugenol had no effect on decreasing glucose rise or blocking this secondary stress response during the experimental handling.

Despite a lack of significance ($P>0.05$), the small differences between T2, T3 and T4 showed a discreet tendency of muscular glycogen increase for T2 to T4. This fact is likely due to attenuation of the metabolic response as a result of the anesthetic effect that promoted lesser muscular activity of the fish. Lower muscular glycogen values after recovery should be due to the absence of feeding during the experiment.

The increase of glucose caused by the endocrine stress response in Nile tilapia was followed by other metabolic responses. Among these was the rise of plasma lactate concentration. This is a consequence of catecholamine release from chromaffin cells through the Brain Sympathetic Chromaffin-Axis (BSC-Axis), and is also an outcome of the intense anaerobic muscular activity (Van Raij *et al.*, 1996). In the treatments subjected to handling or anesthesia, the observed muscular activity was higher compared to the control groups, although at different intensities. This is probably the reason for the lactate concentrations determined in handling (T2), and handling plus anesthesia treatment (T3). In addition, the level of lactate after deep anesthesia (T4) was even higher. Since the eugenol concentration used in T4 was enough to fish quickly reach deep anesthesia, the lactate levels should be lower as a consequence of the decreased muscular activity. This, however, was not observed, and the lactate recovery was longer in this case. This finding was in favor of the assumption that the BSC-Axis may be more relevant in this case than the mechanisms related to glucose release. One option that must be considered is the increase of lactate under anesthesia or handling can be due to a reduction of available oxygen in consequence of the impairment in the intake, transport and diffusion of the oxygen mechanisms (Hochachka, 1980; Lewis *et al.,* 1985). Another possibility is that lactate increase is due to the pharmacological effect from anesthesia. In this case there is a decrease of the heartbeats and the blood flow through the gills (Randal, 1962). Such physiological disorders might combine with metabolic hypoxia and result in lactate production (Iversen *et al.,* 2003). In fact, because T4 result is the highest plasma lactate concentration we hypothesize a combination of all mechanisms may be working together for the lactate increases.

Responses in the nitrogen metabolism may be inferred from changes in plasma ammonia. The increased levels of ammonia both in the handling and the anesthesia groups reflect a continuity of protein catabolism that appears to be preferential among fishes, even in stressful conditions. As a result, ammonia is the most important output (Mommsen *et al.,* 1999). The highest levels were observed in T2, T3 and T4. This is highly suggestive that the nitrogen catabolism was increased and unaffected by any of the eugenol concentrations. Therefore, these results appear to be a consequence of an increase of the metabolic demands to deal with handling rather than effects of eugenol. Another potential reason for high levels of plasma ammonia should be impairment in the gill function, associated

with ammonia excretion. Anesthetic effects were first observed when the fish had a partial or total depression of nervous activities. At this time, cardio-respiratory functions and opercular movements decreased resulting in alterations of the internal biochemical balance and consequent ammonia increase. Gill diffusion of oxygen was probably impaired due to eugenol exposures. This could contribute to an increase of protein catabolism and consequently to the increase of plasma ammonia, particularly in fish submitted to handling plus anesthesia treatments (T2, T3 and T4).

Plasma ion balance and plasma protein concentration were not affected in Nile tilapia. These results were expected because the trials were conducted in short-term exposures. Different results have been reported to fish subjected to hauling and transport (Carneiro and Urbinati, 2001). These procedures are very acute and long stressors. Other factors such as adverse water quality, physical injury and water temperature changes, usually associated with these fish-farming stressors (hauling and transport), may contribute to alter ions balance. By comparison, ion balance appears to be altered only in long-term stressing conditions.

In conclusion, handling initiated the typical biochemical responses to stress in Nile tilapia, which were not affected by the presence of eugenol. Eugenol resulted in easier handling of tilapia, and it did not result in additional stress that could contraindicate its use. However, gill ammonia excretion and oxygen changes were likely impaired by eugenol at the highest tested concentration. Under the condition of our experiments, handling of Nile tilapia may be facilitated through the use of short-term exposures to eugenol at concentrations below 80 mg L^{-1}.

Conclusion

Based on the results of this study, eugenol appears to be a safe anesthetic for use in this species.

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