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Anesthetic properties of *Ocimum gratissimum* essential oil for juvenile matrinxã

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ABSTRACT. The matrinxã fish is suitable for Amazonian aquaculture, exhibiting positive characteristics such as good growth and feed conversion ratio. However, it is a very active fish and must be anesthetized for handling. The present study evaluated the use of *Ocimum gratissimum* essential oil (EO) as anesthetic for juvenile matrinxãs. A first experiment assessed the induction time to anesthesia of 7 concentrations (20, 30, 40, 50, 60, 70 and 80 mg L^{-1} of *O. gratissimum* (EO). A second experiment investigated the physiological response to the anesthetic through analysis of tissue and blood parameters collected 0h (T0) and 24h after (T24) the experimental protocols that were applied to 4 groups: control, handling without *O. gratissimum* (EO) and handling with two EO levels. In the first experiment, fish were anesthetized within 10 min in the lowest EO concentration (20 mg L^{-1}), and recovered within 2 min. As concentrations increased times to induction to anesthesia decreased from 10 min to 1 min (approximately). In the second experiment, plasma lactate, glucose and ammonia increased in the treatments involving handling and EO concentrations. After 24h, fish had recovered from the experimental procedures, and no mortality was observed in the next 30 days. *Ocimum gratissimum* was shown to be a suitable anesthetic for matrinxãs, and causes minimum stress to fish in the concentrations and exposure time applied.

Keywords: fish, handling, *Brycon amazonicus*, anesthesia.

Propriedades do óleo essencial de *Ocimum gratissimum* como anestésico para juvenis de matrinxã

RESUMO. O matrinxã é uma espécie de importância para a piscicultura na Amazônia, já que exibe crescimento e conversão alimentar desejáveis em cultivos comerciais. Entretanto, é um peixe bastante ativo e deve ser anestesiado na execução de práticas de manejo. O presente estudo avaliou as propriedades do óleo essencial de *Ocimum gratissimum* como anestésico para juvenis de matrinxã. Um primeiro experimento avaliou o efeito anestésico de 7 concentrações (20, 30, 40, 50, 60, 70 e 80 mg L^{-1}) do óleo essencial no tempo de indução à anestesia. Um segundo estudo investigou respostas fisiológicas do matrinxã ao óleo essencial utilizado como anestésico através análises de parâmetros sanguíneos e teciduais coletados 0h e 24h após os protocolos experimentais, aplicados em 4 grupos de peixes: controle, manuseio sem anestésico, manuseio em duas concentrações do óleo essencial. No primeiro experimento os peixes foram anestesiados em menos de 10 min e recuperados em torno de 2 min. Os tempos de indução à anestesia foram descrescentes de acordo com o aumento das concentrações até 80 mg L^{-1}. No segundo experimento, os níveis de lactato, glicose e amônia plasmática aumentaram nos tratamentos de manuseio e exposição as concentrações do óleo essencial. Depois de 24h os peixes se apresentaram recuperados dos protocolos experimentais e não foi observada mortalidade até 30 dias depois. O óleo essencial de *Ocimum gratissimum* é um anestésico de origem natural adequado para juvenis de matrinxã, proporcionando ainda segurança aos trabalhadores com mínimo estresse aos peixes nas concentrações e tempo de exposição testados.

Palavras-chave: peixe, manejo, *Brycon amazonicus*, anestesia.

Introduction

The matrinxã, *B. amazonicus*, is an omnivore fish suitable for Amazonian aquaculture, exhibiting good growth and feed conversion ratios under complete and artificial feeding. In less than one year, matrinxãs can reach the commercial size of 1.5 kg (Barbosa, Moraes & Inoue, 2007). However, because this fish is very active and difficult to handle, accidents can occur during its management, especially when this involves delicate procedures

such as the use of electronic devices or sharp and pointed tools (e.g. blades and needles). Thus, matrinxãs need to be anesthetized before handling, thereby ensuring not only fishery workers' safety but also fish integrity, which is important because injuries can facilitate parasitic attack and even death (Inoue, Santos, & Moraes, 2003; Roubach, Gomes, Fonseca & Val, 2005; Tort, Puigcerver, & Crespo, 2002).

Recent studies tested *O. gratissimum* essential oil as fish anesthetic (Benovit et al., 2012; Silva et al., 2012, 2015). The main component of this oil, eugenol, is widely used as a natural fish anesthetic (Barbosa et al., 2007). *O. gratissimum* essential oil has promising potential as an ingredient for natural fish farming products in Brazil. *O. gratissimum* is an aromatic plant from Asia and Africa (Lorenzi & Matos, 2002), but widely distributed in tropical regions with abundant sunshine (Paton, Harley, & Harley, 1999). This sub-spontaneous plant grows throughout Brazil, and is used for cooking and treating anxiety, coughing and vomiting (Lorenzi & Matos, 2002). A number of studies found that *O. gratissimum* can be used against microorganisms such as *Staphylococcus aureus*, *Bacillus spp*, *Pseudomonas aeruginosae*, *Klebisiella pneumoniae*, *Proteus mirabilis* and *Leishmania amazonensis* (Matasyoh et al., 2007; Ueda-Nakamura, Mendonça, Diaz, & Korehisa 2006) and has insecticidal properties (Ogendo et al., 2008).

Despite the current knowledge of *O. gratissimum* essential oil in fish, more investigations are needed to evaluate the use of this product as an anesthetic for matrinxãs. According to Iwama and Ackerman (1994), fish handling without anesthesia may be preferable to anesthetizing fish with inadequate anesthetic levels and exposure time. Anesthetics may be contraindicated for certain fish species and cause adverse side effects. Therefore, the effects of different anesthetics must be tested on as many fish species as possible. In this respect, the present study evaluated the anesthetic effects of the essential oil of *O. gratissimum* on matrinxãs farmed in the Amazon. Time for anesthesia induction and physiological changes in matrinxã were determined after exposure to essential oil.

Material and methods

Green biomass production, essential oil extraction and characterization

The *O. gratissimum* plants used for essential oil extraction were raised for one year in an experimental field of the Western Amazon Division of the Brazilian Agricultural Research Corporation (Embrapa) in Manaus. Plant shoots in the reproductive phase were cut and shade dried to constant weight. The dried plants were taken to the laboratory for essential oil extraction by hydro distillation for 4h in a Clevenger-type apparatus. The composition and chemical characterization of the essential oil was determined by gas chromatography at Embrapa Food Technology, in Rio de Janeiro State, Brazil.

Experiment I: Anesthetic induction time

Juveniles purchased from a fish farm were stocked in a 2 m^3 tank at the National Amazon Research Institute (INPA) facilities, in Manaus, Amazônas State, Brazil. The holding tank was continuously supplied with air and water from a well (temperature 27.7 ± 0.2°C; oxygen 4.2 ± 0.9 mg L^{-1}; pH 5.7 ± 0.2; conductivity 24 ± 0.5 uS cm^{-1}) . Fish were fed commercial pellet ration containing 28% crude protein for 2 months, provided close to satiation twice daily. Feeding was stopped 24h before the experiment.

O. gratissimum essential oil was diluted in ethanol (1:20), and the 0.5% dilution produced (EO) was applied at concentrations of 20, 30, 40, 50, 60, 70 and 80 mg L^{-1} in glass aquaria filled with 4 L of water. One previous test in the experiental facilities established 20 mg L^{-1} as the minimum EO concentration to anesthetize juvenile matrinxãs in less than 10 min. Three aquaria were used to test each concentration, and the tests were performed from the lowest to highest concentration.

One fish was introduced sequentially in each of the 3 aquaria, and anesthetic induction time was recorded in seconds, using one stopwatch per aquarium. Fish were considered anesthetized when they displayed total loss of equilibrium and inability to regain the upright position (Inoue et al., 2003; Woody, Nelson & Ramstad, 2002). The anesthetized fish were measured and transferred to another 2 m^3 holding tank, continuously supplied with water and air. The 3 aquaria used to test each anesthetic concentration were washed and filled with clean water and the following anesthetic concentration to be tested.

Fish were kept and fed in the holding tank for another month before they were counted to calculate survival rate, and then discarded.

Experiment II: Physiological responses

The physiological response to the anesthetic was tested in 180 juvenile matrinxãs (192 ± 46 g; 23.6 ± 1.8 cm) distributed into 12 floating cages (1 m^3) in a 2 ha artificial lake near the Embrapa Western Amazon facilities (02º 53' 12.55 S; 59º 56' 24.70 W),

in Manaus. Fish were fed once a day for one month, with commercial pellet ration containing 28% crude protein. Feeding was stopped one day before the experiment.

The cages were randomly arranged for the 4 treatments with 3 repetitions each. In the control treatment (C), the sampled fish were not anesthetized or handled in any way. Fish from the handling group (H) were transferred from the cage to a 20-L bucket for 10 min., to simulate handling and crowding, and then returned to the cage. The groups subjected to both handling and anesthesia (HA) and handling and deep anesthesia (HDA) were subjected to similar experimental procedures, except for the the addition of 20 and 60 mg L^{-1} EO to the buckets, respectively. Tissues and blood samples were taken from 3 fish in each cage after they were moved from the buckets to the cages (T0). Three other fish from each cage were sampled 24h later (T24). The remaining fish were fed and observed for another month. Then they were counted and discarded.

Fish sampled from each treatment were killed by a sharp blow to the head and their blood harvested from the caudal vein using 3 mL syringes (rinsed with 10% EDTA). Fragments of liver and white muscle were collected for glycogen determination (Bidinotto, Moraes, & Souza, 1997).

Blood samples were used for hematocrit (Goldenfarb, Bowyer, Hall & Brosious, 1971) and total hemoglobin determination (Drabkin, 1948) and red cell count (Lima, Soares, Greco, Galizzi, & Cançado, 1969). In addition, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated (Lima et al., 1969).

After centrifugation of blood aliquots (14400 G x 3 min.), plasma was used to determine glucose (Trinder, 1969), total ammonia (Gentzkow & Masen, 1942), lactate (Harrower & Brown, 1972), chloride American Public Health Association (APHA, 1980), total protein (Lowry, Rosebrought, & Farr, 1951) Na and K levels by flame photometry (Digimed, DM-61 model).

Data analyses

Data on anesthetic induction were evaluated by polynomial regression ($p < 0.05$). Data on both anesthetic induction and physiological parameters were submitted to one-way ANOVA followed by tukey's test for multiple comparisons ($p < 0.05$).

Results and discussion

The major components were eugenol, 1.8 cineole and beta-selinene (Table 1). Eugenol content was lower than observed by Silva et al. (2012). This may change according to plant specific genetic characteristics and local conditions. Usually when the major component decrease minors increase. In general, there is no uniform essential oil composition. This may change when oil is extract from different parts of the same plant, farming practices, and extraction method. Difference in the compositon is known as the oil components are plant metabolism products so variable according plants life cicle. Some components transformations to anothers may take place, and even after oil extraction. Reactions among the different oil components may occur as interactions among oil and environmental factors (light, enzymes and the container) are dynamics (Luz, Ehlert, & Innecco, 2009).

Table 1. Main components of *O. gratissimum* essential oil. Retention Index[1].

RI[1]	Component	%
938	alpha-pinene	1.0
975	sabinene	0.7
979	beta-pinene	2.8
991	myrcene	0.7
1032	1,8-cineole	28.2
1038	*cis*-ocimene	3.7
1049	*trans*-ocimene	0.0
1097	linalol	1.3
1166	delta-terpineol	0.4
1176	4-terpineol	0.4
1188	alpha-terpineol	1.1
1357	eugenol	43.3
1381	beta-bourbonene	0.9
1389	beta-elemene	0.8
1415	beta-caryophyllene	3.7
1450	alpha-humulene	0.6
1477	gamma-muurolene	0.9
1482	beta-selinene	5.5
1490	alpha-selinene	1.7
1513	7-*epi*-alpha-selinene	0.4
Total		98.1

The movement of fish was always intense when they entered the anesthetic bath. After a few seconds, they were less agitated and started losing equilibrium. Total loss of equilibrium and inability to regain the upright position was achieved in less than 10 min. Anesthetic induction time was longer in the treatment using the lowest EO concentration of 20 mg L^{-1} than in that using 30 mg L^{-1}, and data from both exhibited higher standard deviations than the other treatments (from 40 to 80 mg L^{-1}). Treatments testing EO concentrations from 40 to 80 mg L^{-1} showed similar induction time for anesthesia (Figure 1).

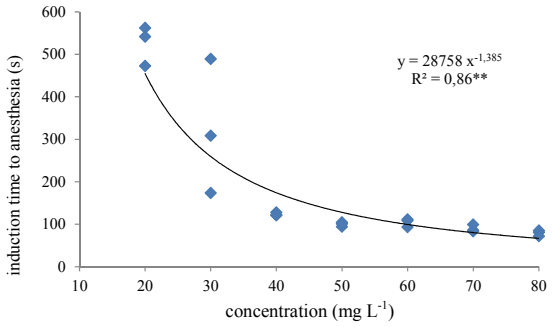

Figure 1. Anesthetic effect of *Ocimium gratissimum* essential oil in juvenile matrinxa. Times (in seconds) were measured to induce total loss of equilibrium and inability to regain the upright position.

The equation developed to predict time for anesthesia induction in juvenile matrinxãs with 20 to 80 mg L^{-1} of EO was $Y = 28758\ c^{-1.385}$ ($R^2 = 0.86$; $F = 428.641$), where Y is induction time to anesthesia in seconds, and c is EO concentration in mg L^{-1}. In general, a fish anesthetic should provide anesthesia in less than 3 min., (Marking & Meyer, 2011). In this work fish weight (g) and length (cm) were easily measured in the anesthetized fish. After they were returned to anesthetic-free clean water, fish recovered from anesthesia in less than 2 min. as suggested Marking and Meyer (2011), and time to equilibrium reestablishment was similar for all treatments. Fish restarted feeding nearly 6h after the experiment. No fish mortality was observed even one month after the tests.

Hematocrit and MCHC did not differ among groups at T0 or T24 (Table 2). However, hemoglobin and RBC were lower at T24 than T0. MCV and MCH values were higher at T24 than in T0 (Table 2).

Plasma potassium and protein levels were maintained, as were muscle and liver glycogen. Plasma sodium and chloride had no statistical differences (Table 3).

At T0, matrinxãs submitted to HDA exhibited the highest levels of plasma glucose ($F = 14.007$) and ammonia ($F = 9.353$) (Table 3). At T0, plasma lactate increased due to handling and anesthetic concentrations compared to the experimental control ($F = 2.420$). Recovery from the experimental procedures at T24 was similar among treatments, irrespective of handling or essential oil application. No fish mortality was observed one month after Experiment II.

Certain management practices cause acute stress in farmed fish, compromising their equilibrium with the environment and triggering physiological responses. When the stressor is applied for long periods and its intensity is high, it may compromise production, triggering diseases and mass fish mortality (Iwama, Afonso, Todgham, Ackerman, & Nakano, 2004). Nevertheless, stressing procedures such as handling are necessary for fish farming.

Table 2. Blood parameters (mean ±sd) of matrinxãs 0 and 24 hours after experimental procedures involving handling (H), handling associated to anesthesia (HA) or to deep anesthesia (HDA). Anesthetic was *O. gratissimum* essential oil in concentrations of 20 mg L^{-1} (H.A.) and 60 mg L^{-1} (H.D.A.) for 10 min. Fish from control were only sampled.

Treatment	hematocrit (%)	hemoglobin (g dL^{-1})	red cell count (millions mm^{-3})	MCV (μmm^3)	MCH (pg $cell^{-1}$)	MCHC (g dL^{-1})
			0h after handling			
Control	44±6	15.1±1.5[a]	2.6±0.4[a]	171±28[a]	60±10[a]	36±5
H	43±7	16.0±2.0[a]	2.5±0.3[a]	175±24[a]	64±12[a]	37±6
HA	43±7	16.0±2.0[a]	2.2±0.3[a]	194±27[a]	75±12[a]	39±7
HDA	44±5	17.0±2.0[a]	2.3±0.5[a]	211±54[a]	81±22[a]	38±5
			24h after handling			
Control	39±4	12.0±5.1[b]	2.1±0.3[b]	184±20[a]	57±22[a]	32±12
H	39±6	15.0±2.0[b]	1.7±0.2[b]	224±42[a]	89±11[a]	40±6
HA	42±5	16.0±3.0[b]	2.0±0.2[b]	204±30[a]	78±17[a]	38±7
HDA	41±3	17.0±2.0[b]	1.6±0.2[b]	257±32[b]	108±23[b]	42±6

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration. Different letters in a column indicate statistical differences among the treatments for a same period (Anova, $p < 0.05$).

Table 3. Plasma parameters (mean ± sd) of matrinxãs immediately after and 24 hours after experimental procedures involving handling (H), handling associated to anesthesia (HA) or to deep anesthesia (HDA) with *O. gratissimum* essential oil.

Treatment	glucose (mg dL^{-1})	lactate (mg dL^{-1})	Na (mEq L^{-1})	Cl^- (mEq L^{-1})	K (mEq L^{-1})	protein (g dL^{-1})	ammonia (µmol mL^{-1})
			0h after handling				
Control	52±10[a]	12±4[a]	128±16	116±1.5	1.3±0.5	5.1±2.0	2.6±0.4[a]
H	62±19[a]	58±4[b]	139±31	112±3.2	1.9±0.3	4.4±0.6	3.4±0.9[a]
HA	66±13[a]	77±22[b]	122±14	117±2.1	3.5±0.3	4.3±0.4	3.5±1.1[a]
HAD	79±16[b]	79±28[b]	110±13	120±6.4	2.6±1.8	4.3±0.4	4.1±1.4[b]
			24h after handling				
Control	30±5[a]	28±5[a]	127±15	109±6.3	3.3±0.3	4.3±0.6	2.4±0.4[a]
H	45±15[a]	31±12[a]	110±20	108±9.5	3.9±0.3	5.4±2.4	3.3±1.4[a]
HA	47±10[a]	21±14[a]	101±2	112±8.3	5.0±0.3	4.4±0.4	3.0±0.8a
HDA	38±8[a]	36±6[a]	116±18	110±5.2	3.9±0.3	4.2±0.5	3.2±1.3a

Different letters in a column indicate statistical differences among the treatments for a same period (Anova, $p < 0.05$).

In Experiment I, the concentrations of *O. gratissimum* essential oil were found to be sufficient to anesthetize 120 g of juvenile matrinxãs, facilitating their biometric assessment. Concentrations above 40 mg L^{-1} EO were more efficient to cause total loss of fish equilibrium and inability to regain the upright position in approximately 1.5 minutes. The use of 60 mg L^{-1} EO provided faster anesthesia, but Experiment II showed that this concentration was more stressful to the fish.

The response of fish to anesthetics such as MS 222 (tricaine methanosulfonate), clove oil, metomidate, benzocaine, carbonic gas, and phenoxyethanol are described, but their effects as stress reducers are sometimes contradictory (Iversen, Finstad, Mckinley, & Eliassen, 2003; Pirhonen & Schreck, 2003; Tort et al., 2002; Wagner, Singer, & Mckinley, 2003). In the present study, the stressor used (fish transfer from the cage to the buckets and then back to the cage) simulated a practice adopted in field conditions and was adverse enough to trigger a stress response. This was observed at T0, by increasing plasma lactate in the experimental treatments. Plasma glucose and ammonia levels also increased after experimental handling, but this increase was statistically significant only in HDA. An increase in blood parameters occurs immediately after the nervous system receives one or more adverse stimuli, activating two metabolic axes, the CPI (cerebrum-pituitary-interrenal cells) and the CSC (cerebrum, sympathetic chromaffin cells). Cortisol and catecholamine are released in the blood stream initiating metabolic processes for extra energy production allowing fish to escape or adapt to the new condition (Iwama et al., 2004). Therefore, the increase in plasma glucose and ammonia observed in juvenile matrinxãs from the HDA group suggests they entered a stress condition with a high level of anesthesia. Therefore, with 10 min exposure time, the ideal concentration of EO as anesthetic for matrinxãs is between 20 and 60 mg L^{-1}.

An increase in plasma glucose is an important indicator of fish stress under field conditions (Hattingh, 1976). Therefore, the increase in plasma glucose in fish from the HAD group (Experiment II) reinforces the stress response produced by the highest EO concentration used. The stress response is mediated by cortisol (Iwama et al., 2004; Mommsen, Vijayan, & Moon, 1999), but the direct determination of cortisol under field conditions is in general unfeasible in Brazil. Other works were done using plasma glucose as stress indicator in fish anesthesia.

Roubach, Gomes, and Val (2001) reported an increase in plasma glucose in matrinxãs anesthetized with MS 222 at concentrations above 150 mg L^{-1} for 10 min. In another study, the plasma glucose of matrinxãs decreased after exposure to 60 mg L^{-1} of benzocaine for 10 min., (Inoue, Hackbarth, & Moraes, 2004). Tilapias and matrinxãs exposed to 80 and 60 mg L^{-1} of eugenol for 10 min., had decreased stress (Barbosa et al., 2007; Deriggi, Inoue, & Moraes, 2004). Studying juvenile tambaquis, Roubach et al. (2005) also observed stress reduction after exposure to 135 mg L^{-1} eugenol for 10 min. Other studies have not found that tilapia, tambaqui and matrinxã were stressed by anesthesia with eugenol (Barbosa et al., 2007; Deriggi et al., 2006; Roubach et al., 2005). Other EO concentrations and times of exposure have to be studied in matrinxas.

High plasma ammonia is another metabolic response mediated by cortisol (Mommsen et al., 1999). In the present study, another fact may have contributed to the rise of plasma ammonia in fish from the HDA group at T0. In anesthetic baths, fish first increase and then decrease their operculum movements, and with a change in water flow through the gills, nitrogen excretion is impaired, thereby increasing plasma ammonia levels. Blood water content likely increased 24h after the experimental procedures were applied (T24), given that hemoglobin and red cell count decreased. Also in the HDA group, MCV and MCH increased at T24. A decrease in sodium and chloride levels could explain increases blood water content (McDonald & Milligan 1997), but this was not found in the present study.

The anaerobic metabolism of plasma lactate increases in fish submitted to experimental handling (Hochachka, 1980). Inoue et al. (2004) observed a decrease in plasma lactate in matrinxãs anesthetized with 60 mg L^{-1} benzocaine or with 600 mg L^{-1} phenoxyethanol for 10 min. At T0, *O. gratissimum* essential oil increased plasma lactate in matrinxã, although differences in the concentrations tested were not significant. Some degree of respiratory deficit may have occurred during anesthesia, but the fish recovered at T24. In addition, the 10 min. of exposure to *O. gratissimum* essential oil was probably too short to affect plasma lactate, and this exposure time therefore seems to be adequate to anesthesize matrinxãs.

Conclusion

O. gratissimum essential oil can be used as anesthetic for matrinxãs. Although this oil contains other components besides eugenol, they apparently do not cause undesirable side effects. After 24h, fish recovered from stress of handling and anesthesia, and the procedures applied did not cause fish mortality for a 30-day period. The use of *O. gratissimum* EO at concentrations from 20 to 60 mg L^{-1} as anesthetic, with a 10-min. exposure time, is safe for fishery workers to handle and causes minimum stress during matrinxã handling.

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