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Anesthesia and transport of fat snook *Centropomus parallelus* with the essential oil of *Nectandra megapotamica* (Spreng.) Mez

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This study analyzed the chemical composition and anesthetic potential of essential oil (EO) of *Nectandra megapotamica* in fat snook (*Centropomus parallelus*). For the extraction of EO by hydrodistillation, leaves were separated in young (EO-Y) or old (EO-O), and the chemical composition of the EOs was determined by CG-MS. The anesthetic potential was assessed by the evaluation of induction and recovery time of anesthesia and stress response from anesthesia and transport. Three experiments were carried out: i) four different concentrations of each EO were tested to evaluate anesthesia induction and recovery time; ii) two concentrations of EO-O were tested for the evaluation of its effects on stress parameters (glucose, lactate, and Na^+ and K^+ plasma levels) caused by anesthesia; and iii) fish were transported in plastic bags, supplied with two concentrations of EO-O for the evaluation of water quality and mortality. All experiments were performed on fish acclimated to 0 and 33 ppt salinity. The main constituents of the Y and O-EOs were bicyclogermacrene (46.5/34.6%), α-pinene (26.8/26.2%), β-pinene (7.9/12.3%), and germacrene D (9.6/9.1%). Mild sedation was achieved at 30 μL L^{-1}(1.3-3.2 min) and deep anesthesia at 150 μL L^{-1} (5.6-8.0 min) with both EOs. The recovery time ranged from 1-10 min. The EO-O was not able to avoid the stress of anesthesia evidenced by elevated glucose and lactate plasma levels observed in all groups. Plasma levels of Na^+ and K^+ were not significantly affected by treatments. During transport, the use of EO-O did not prevent deterioration in water quality and the post-transport mortality. In conclusion, the EO of *N. megapotamica* has anesthetic activity in fat snook, but it was not able to prevent the stress of anesthesia and transport.

Este estudo analisou a composição química e o potencial anestésico do óleo essencial (OE) de *Nectandra megapotamica* em robalos-peva (*Centropomus parallelus*). Para a extração do OE por hidrodestilação, as folhas foram separadas em jovens (OE-J) ou velhas (OE-V) e a composição química foi determinada por CG-EM. O potencial anestésico foi acessado através da avaliação do tempo de indução e recuperação da anestesia e resposta ao estresse do procedimento anestésico e transporte. Foram realizados três experimentos: em primeiro lugar, quatro concentrações diferentes de cada OE foram testadas para avaliar o tempo de indução à anestesia e de recuperação; em segundo lugar, duas concentrações do OE-V foram testadas para avaliar os efeitos sobre os parâmetros de estresse (níveis plasmáticos de glicose, lactato, Na^+ e K^+) causados pelo procedimento anestésico; em terceiro lugar, os peixes foram transportados em sacos plásticos com duas concentrações do OE-V para avaliação da qualidade da água e mortalidade. Todos os experimentos foram realizados em peixes aclimatados à salinidade zero e 33. Os constituintes majoritários do OE-J e OE-V foram: biciclogermacreno (46,5/34,6%), α-pineno (26,8/26,2%), β-pineno (7,9/12,3%) e germacreno D (9,6/9,1%). Sedação leve foi alcançada com 30 μL L^{-1}(1,3-3,2 min) e anestesia profunda a partir de 150 μL L^{-1} (5,6-8,0 min) com ambos OEs. O tempo de recuperação variou entre 1-10 min.O OE-V não foi capaz de evitar o estresse do procedimento anestésico,

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evidenciado pelos elevados níveis plasmáticos de glicose e lactato observados em todos os grupos. Os níveis plasmáticos de Na^+ e K^+ não foram significativamente afetados pelos tratamentos. Durante o transporte, o OE-V não impediu a deterioração da qualidade da água e a mortalidade pós-transporte. Concluindo, o OE de *Nectandra megapotamica* apresenta atividade anestésica em robalos-peva, mas não foi capaz de evitar o estresse do procedimento anestésico e transporte.

Key words: Fish, Ionoregulation, Sedation, Stress, Terpenoids.

Introduction

Anesthetics are used in modern aquaculture management to minimize stress or physical damage caused by handling, transport, and more invasive procedures that may cause poor performance, immunosuppression, and increased mortality in fish (Gray *et al.*, 2011). A variety of anesthetics such as tricaine methanesulphonate (MS-222), 2-phenoxyethanol, quinaldine, benzocaine, and metomidate have been used for this purpose (Ross & Ross, 2008). However, the use of some anesthetics has been limited or rejected due to undesirable side effects for fish and humans (Palic *et al.*, 2006). Alternatively, several studies using plant derivative compounds, such as essential oils (EOs) from *Eugenia*, *Mentha*, and *Lippia* species, have demonstrated that these natural products are effective for fish sedation and may have benefits over traditional methods, including lower cost and higher safety (Cunha *et al.*, 2010a; Gonçalves *et al.*, 2008; Palic *et al.*, 2006).

The genus *Nectandra* Rol. Ex Rottb (Lauraceae) includes more than 100 recognized species of canopy trees mainly distributed from Amazon to Southern Brazil, Argentina, Paraguay, and Uruguay (Alves & Sartori, 2009). *Nectandra megapotamica* (Spreng.) Mez is popularly known as "canela-preta" and its barks have been used in folk medicine as anti-rheumatic and analgesic (Santos Filho & Gilbert, 1975) and its leaves have been used as sedative (Alves *et al.*, 2008). Previous pharmacological investigation using mice and rats have showed analgesic and anti-inflammatory activities for the bark hydroalcoholic extract of this species (da Silva *et al.*, 2004). Additionally, studies have showed that the ethanolic extract from the trunk bark presents antioxidant and antimicrobial activities (Garcez *et al.*, 2009), while for the leaf essential oil, literature describes anti-inflammatory and antitumor activities (Apel *et al.*, 2006).

Snooks (*Centropomus* spp.) have been recognized as fish with great potential for aquaculture due to their fast growth, highly efficient food conversion ratios and energy utilization (Alvarez-Lajonchere & Tsuzuki, 2008). In the production process, the transport of juveniles is one of the most important steps in the management and marketing of fish. This practice inevitably involves stress and may cause a large-scale mortality. Several studies have shown the positive effect of anesthetics during transport of many fish species (Inoue *et al*., 2005; Iversen *et al*., 2009; Pramod *et al*., 2010).

The objective of the present study was to determine the anesthetic potential of the EO of *N. megapotamica* evaluating the induction and recovery times from anesthesia as well as its effect on biochemical and osmoregulatory parameters related to stress of anesthesia and transport in fat snook, *Centropomus parallelus*. Additionally, the chemical composition of the EO was analyzed in order to determine the possible substances responsible for the detected activity.

Material and Methods

Experimental fish. Fat snook juveniles were purchased from Pandini Fish Culture (São Mateus, ES, Brazil) and transported to the Laboratory of Fish Culture at Universidade Federal do Espírito Santo (UFES - Aracruz), where they were gradually acclimated to freshwater (FW) (reducing 10 ppt each day) or maintained in 33 ppt (seawater (SW) salinity in the tanks where fish were raised) for 10 days. They were kept in continuously aerated tanks of 500 L (small fish) and 1000 L (large fish), fed with commercial pellets NRD 1.2 (Inve®) three times a day and fasted 24 h before the experiments. Data on water quality parameters [FW / SW] were: conductivity (mS), 0.183 - 5.3 / 49.6 - 55.2; salinity (ppt), 0 / 33.1 - 35.2; temperature (ºC), 23.1 - 25.1 / 24.2 - 27.0; dissolved oxygen ($mg\ L^{-1}$), 7.62 - 7.87 / 7.15 - 7.62. Data were measured using an YSI 85 multiparameter instrument.

Plant material. The plant *Nectandra megapotamica* was collected from native forests of Santa Maria, State of Rio Grande do Sul, Southern Brazil. The aerial parts of the plant were collected in November 2010 and identified by Prof. Dr. Solon Jonas Longhi from the Department of Forestry Engineering, Universidade Federal de Santa Maria (UFSM) . A voucher specimen (SMDB N. 13107) was deposited in the herbarium of the Departamento de Biologia, UFSM. The leaves were classified into young (Y) and old (O) samples considering the arrangement of leaves on the branches, with nodes and internodes at different cycles of tree growth as control (Penfold & Willis, 1961). Size, texture, color and particle deposition on the upper surface of the sheet were also considered.

Essential oil extraction. The young and old leaves were submitted separately to hydrodistillation using a Clevenger type apparatus for 3 h. In this method, the distillate is collected in a graduated glass tube and the aqueous phase is automatically reused into the distillation flask (Council of Europe, 2007). The EO from young leaves (EO-Y) and old leaves (EO-O) was stored at -4°C in amber glass bottles.

Volatile oil analysis and identification of constituents. EO samples were analyzed by gas chromatography-mass spectrometry (GC-MS), using an Agilent 6890N chromatograph coupled with an Agilent 5973 mass selective detector operating at 70 eV. The analyses were carried out using a HP-5MS capillary

column (30 m x 0.25 mm x 0.25 μm), injecting 1.0 μL of a solution at 1.0 μL mL^{-1} of EO in hexane in split inlet mode (1:100). The carrier gas (He) flow rate was 1.3 mL min^{-1}, injector and detector temperatures were set at 250 and 320°C, respectively. The oven temperature was programmed from 40-320°C at 4°C min^{-1}. The identification of the individual EO components was accomplished by comparison of the Kovats retention index with literature data and by matching mass spectral data with those available in the system (Adams, 2001; NIST, 2002). For quantitative analysis, the percentage composition of the EO samples was computed by integrating the peak area of the chromatograms.

Anesthesia induction and recovery. Snook fingerlings (0.49±0.03 g and 3.65±0.08 cm, *n* = 8 per treatment) were transferred to glass aquaria contained 100 mL of FW or SW and the EOs (Y or O) of *Nectandra megapotamica* at concentrations of 30, 150, 300 and 450 μL L^{-1} (equivalent to 24, 120, 240 and 360 mg L^{-1}, respectively because the density of this EO was approximately 0.80). EO samples were first diluted in ethanol (1:10). Control experiments were performed using aquaria that contained only ethanol at concentrations equivalent to the dilutions used for 300 and 450 μL L^{-1} EO (2700 and 4050 μL L^{-1}). To evaluate the time required for anesthesia induction, fingerlings were evaluated individually, and each fingerling was used only once. The stages of anesthesia were evaluated according to the method of Stoskopf (1993) where the following stages (S) can be observed: S1, slight loss of reactivity to visual and tactile stimuli; S2, partial loss of equilibrium; S3, total loss of equilibrium; S4, reduced opercular movement; S5, minimal opercular movement; REC, recovery of equilibrium and swimming activity.The observation time was 10 min and at the end of this period juveniles were transferred to anesthetic-free aquaria to measure the anesthesia recovery time.

Biochemical and osmoregulatory parameters. Juveniles (37.2±4.03 g and 28.7±3.77 g, for FW and SW adapted fish, respectively; *n* = 6 for group) were anesthetized with 30 and 300 μL L^{-1} EO-O of *Nectandra megapotamica* first diluted in ethanol (1:10) in aquaria containing 1 L of FW or SW. These two concentrations were chosen after the induction and recovery trials. Fish taken directly from the main tank (FW or SW) were used as baseline control values (BC) and fish kept in anesthetic-free aquaria for 10 min (simulating anesthesia procedure) were considered aquarium control (AC). After anesthesia, blood was collected from the caudal vein and glucose levels were determined with the digital Accu-Chek Active (Roche™). The remaining blood was centrifuged at 3000 rpm for 10 min and plasma was then frozen for later analysis. Plasma Na^+ and K^+levels were determined with a flame photometer Analyser 910 (Analyser, São Paulo, Brazil) and plasma lactate levels by means of biochemical kit (Bioclin, K084) using a semi-automatic biochemical analyzer (Model BIO-200F; Bioplus, Brazil). Immediately after blood collection, fish were euthanized by cervical section to remove the gills and muscle for tissue hydration determination. After weighing, the tissues (wet weight) were dried for 24 h at 60°C and then weighed again (dry weight). The loss in weight (water) was expressed as a percentage of the initial wet weight of the sample.

Transportation experiment. Fish were removed from the acclimation tanks and placed in 4 L polyethylene bags with 1 L of FW or SW at loading densities of 10 fish L^{-1}(16.76±0.59 g L^{-1}) for FW and 25 fish L^{-1} (11.16±0.24 g L^{-1}) for SW. The EO-O of *Nectandra megapotamica*, firstly diluted in ethanol (1:10), was added to plastic bags at 15 or 30 μL L^{-1}. Control groups used only water or ethanol at the same concentration used for dilution of 30 μL L^{-1} of EO. The bags were inflated with pure oxygen and then sealed. The experiment was performed in triplicate. The transport was carried out on paved and unpaved roads for 10 h (usual time of transport of fingerlings of this species in Brazil). Water quality parameters (temperature, pH, conductivity, oxygen, and ammonia) were analyzed before and after transport and mortality was observed at times zero (arrival), 12, 60 and 84 h after transport. Total ammonia was determined by indophenol blue method (American Public Health Association, 1998) and other parameters as previously described. Ammonia excretion was calculated according to Gonzales *et al*. (1998).

Statistical analyses. All data are represented as mean ± SEM and were subjected to Levene's test to check homogeneity of variances. Data from the time of anesthesia induction and recovery were subjected to logarithmic transformation to obtain homogeneity between groups and two-way ANOVA followed by Tukey test were performed to test for difference between groups. Data expressed in percentages (mortality) were subjected to arcsine transformation and analyzed by two-way ANOVA followed by Tukey test. Biochemical and osmoregulatory parameters were analyzed by one-way ANOVA. The relationship between anesthetic concentration and time to reach each anesthetic stage or recovery were assessed using Sigma Plot 11.0 software. Significant difference was established at $P < 0.05$.

Results

Volatile oil analysis and identification of constituents. A total of 23 and 21 compounds were identified in the EO-Y and EO-O, accounting for 99.86 - 99.81% of their total composition, respectively. These EOs of *Nectandra megapotamica* were composed largely of monoterpene and sesquiterpene hydrocarbons. The main constituents of the EO-Y and EO-O were bicyclogermacrene (46.5/34.6%), α-pinene (26.8/26.2%), β-pinene (7.9/12.3%), and germacrene D (9.6/9.1%) (Table 1).

Anesthesia induction and recovery. As expected, the increasing concentration of EOs proportionally decreased the time required for sedation and anesthesia induction in most stages, but increased recovery time (Table 2). The greater induction time for 150 μL of EO-Y in comparison with 30 μL cannot be explained by current knowledge. Further studies at molecular level should be performed to clarify this fact. All fish recovered from anesthesia within 30 min and no mortality was registered in the course of the experiment. For anesthesia induction, 30 μL L^{-1} EO-O was able to

induce stages 1-4, but stages 3 and 4 in FW and stage 4 in SW were reached by only 25, 37.5, and 25% of fish, respectively. Fat snooks acclimated to SW and exposed to the same concentration of EO-Y did not reach stage 3 and fish recovered before the end of exposure to this EO. From the 150 μL L^{-1} EO-Y and EO-O all fish reached stage 4 (reduced opercular movement) and minimal opercular movement (stage 5) was reached only by fish exposed to 450 μL L^{-1} EO-Y, in both type of water (Table 2).

There was no significant difference in the time to reach the different stages of anesthesia in FW-adapted fat snook exposed to 30 and 300 μL L^{-1} EO-Y and EO-O. The induction times to stage 3 at 150 μL L^{-1} EO-O and stage 1 at 450 μL L^{-1} EO-O were significantly lower than those using EO-Y at the same concentrations in FW. Tests in SW showed no significant difference between induction time for the different stages with EO-Y or EO-O at 30 μL L^{-1}, while time to reach stage 1 at 150, 300 and 450 μL L^{-1} EO-O and time to reach stage 2 at 300 μL L^{-1} EO-O was significantly higher than using EO-Y. The induction time to stage 3 was significantly lower at 450 μL L^{-1} EO-O than with EO-Y in SW. Recovery time was significantly higher at 150 and 450 μL L^{-1} EO-O in SW and at 450 μL L^{-1} EO-O in FW when compared to the correspondent data of EO-Y (Table 2).

Time to reach the different anesthesia stages at 30 and 150 μL L^{-1} EO-Y was not affected by FW or SW adaptation. In SW, induction times to reach stage 3 were significantly higher than in FW at 300 and 450 μL L^{-1} EO-Y, but time to reach stage 1 at 450 μL L^{-1} EO-Y had a significantly lower induction time. Fat snooks exposed to 150 μL L^{-1} EO-O had significantly higher induction times to stages 1, 2 and 3 in SW than in FW. Recovery time was significantly higher at 300 and 450 μL L^{-1} EO-Y and 150, 300, and 450 μL L^{-1} EO-O in SW than in FW. All fish (FW or SW adapted) exposed to 2700 μL L^{-1} ethanol alone reached the stage 1 within 30 s, but 87.5% showed full recovery even in ethanol after 2 min. Fat snooks exposed to 4050 μL L^{-1} ethanol reached stage 1 within 10 s. Half of the fish reached the stage 3 in 20 s, but after 2 min only 25% of the total remained in this stage up to the end of the observation (10 min). Recovery was immediate in aquaria containing only water.

Table 1. Constituents of the young (EO-Y) and old leaf essential oil (EO-O) of *Nectandra megapotamica*. RI_{lit} = retention index from literature; RI_{exp} = experimental retention index; NI = not identified; a = Adams (2001); b = databank NIST (2002).

Compound		EO-Y		EO-O	
	RI_{lit}	%	RI_{exp}	%	RI_{exp}
α-pinene	931 b	26.82	933	26.19	930
camphene	943 b	0.23	944	0.67	943
β-pinene	973 b	7.95	973	12.30	972
β-myrcene	991 a	1.38	991	2.39	990
limonene	1026 b	2.31	1026	4.61	1026
1,8-cineole	1029 b	0.27	1028	0.56	1028
β-*Z*-ocimene	1037 a	0.06	1037	-	-
β-*E*-ocimene	1050 a	0.43	1048	0.74	1049
terpinolene	1086 b	0.07	1086	-	-
linalool	1097 a	0.28	1099	0.71	1100
δ-elemene	1338 a	0.22	1337	1.13	1338
α-copaene	1377 a	0.50	1376	0.46	1377
NI	-	-	-	0.18	1386
β-cubebene	1390 b	0.11	1390	0.15	1391
β-elemene	1393 b	0.11	1392	0.52	1393
β-caryophyllene	1420 b	1.48	1420	2.39	1421
α-caryophyllene	1455 a	0.37	1454	0.52	1455
germacrene D	1485 a	9.61	1484	9.20	1483
α-selinene	1490 b	0.25	1487	0.34	1488
bicyclogermacrene	1500 a	46.47	1501	34.56	1499
δ-cadinene	1523 b	0.63	1524	0.70	1525
E-nerolidol	1563 a	0.14	1564	-	-
spathulenol	1578 a	-	-	1.06	1580
globulol	1585 a	0.17	1585	0.62	1587
NI	-	0.14	1593	-	-
Total identified		99.85		99.82	
Yield		0.30		0.20	

Biochemical and osmoregulatory parameters. Fish subjected to simulation of anesthesia (AC) and exposed to 30 and 300 μL L^{-1} EO-O in SW showed significantly higher plasma glucose and lactate levels compared to basal control (BC), but groups did not show significant difference between each other. Both parameters were significantly higher in FW-adapted fat snooks anesthetized with 300 μL L^{-1} EO-O when compared to BC group. Plasma glucose levels were significantly higher in SW fish subjected to AC and exposed to 30 and 300 μL L^{-1} EO-O than in FW fish subjected to the same treatments. SW-adapted fat snooks subjected to AC also presented significantly higher lactate levels than FW fish submitted to the same treatment (Table 3).

The different treatments (AC, 30 and 300 μL L^{-1}) did not change significantly the plasma Na^+ and K^+ levels in fish kept at either salinities. Plasma Na^+ levels of fish from BC, AC and 30 μL L^{-1} groups maintained in SW were significantly higher than those of the same groups kept in FW. No significant differences were observed for plasma K^+ levels between fat snooks from either salinities (Table 3). Overall muscle and gill water content was *ca.* 80% and 68-74%, respectively, for all groups and was not affected significantly by treatments or salinity (Table 4).

Transportation experiment. After 10 h transportation, in FW-acclimated groups there was no death on the arrival. Post-transport mortality was low up to the end of 84h and showed no significant difference between groups (Fig 1A). In SW-acclimated fat snooks mortality ranged from 2.6-12% and increased up to 12h, differing statistically from arrival. Post-transport mortality was significantly higher in fish transported with 30 μL L^{-1} EO-O (Fig. 1B). Water quality parameters at the end of transportation and ammonia excretion did not show any statistical differences between treatments for either water types. The range in water temperature, conductivity and pH at the end of transportation (24.97-25.23°C / 27.70-28.66°C, 0.11-0.12 / 54.30-55.66 mS, 6.47-6.67 / 6.66-6.95, respectively for FW / SW) also did not show any statistical differences compared with water before transport (24 / 29°C, 0.134/ 52.7 mS, 7.04 / 7.64, respectivelyfor FW / SW). The dissolved oxygen level before adding pure oxygen for transport in plastics bags was 7.75/ 7.77mg L^{-1} for FW / SW, respectively, and at the end of the transport was supersaturated (higher than 200%) for all groups in both salinities. Total ammonia levels increased significantly

Table 2. Time (in seconds) required for induction and recovery from anesthesia using the essential oil of *Nectandra megapotamica* in fat snook. Stages are according to Stoskopf (1993): S1, slight loss of reactivity to visual and tactile stimuli; S2, partial loss of equilibrium; S3, total loss of equilibrium; S4, reduced opercular movement; S5, minimal opercular movement; REC, recovery of equilibrium and swimming actively; Maximum observation time was 10 min. N = 8 for each concentration tested. §, number of observations lower than 3 (data not used for statistical comparison); *, significantly different from EO-Y in the same salinity; #, significantly different from freshwater with the same essential oil; -, stage not reached by any of the animals.

	EO-Y (μL L^{-1})				EO-O (μL L^{-1})			
	Freshwater							
	30	150	300	450	30	150	300	450
S1	22.5±5.5	47.6±18.1	29.5±5.3	26.8±4.4	28.8±3.5	16.0±1.4	21.5±2.1	16.8±0.9*
S2	76.2±24.2	75.1±16.4	42.7±3.0	35.2±6.2	192.0±49.0	42.3±5.5	36.1±3.4	29.0±1.1
S3	213.7±54.2	295.5±55.0	158.8±25.6	159.3±45.8	370.5±36.4§	123.6±19.2*	214.0±33.8	260.1±34.2
S4	294.7±43.3	449.2±40.3	371.1±54.6	371.1±54.6	420.3±20.6§	337.2±47.0	393.6±35.0	368.7±33.4
S5	-	-	-	322.0±116.3	-	-	-	-
REC	78.0±37.2	334.5±40.8	314.1±74.6	405.1±64.7	35.8±14.8	341.5±78.3	478.1±24.3	910.7±81.8*
	Seawater							
S1	21.0±3.2	16.8±2.1	12.5±1.0	11.2±1.2#	20.1±1.8	26.2±3.2*#	17.7±1.0*	16.3±1.0*
S2	122.0±39.0	79.7±8.7	35.6±2.8	26.5±4.0	123.7±11.4	79.1±5.4#	49.1±5.0*	32.3±3.1
S3	-	437.7±34.5	386.5±46.5#	365.0±24.8#	349.1±47.2	347.4±38.9#	260.1±16.5	283.2±17.1*
S4	429.4±36.4	481.3±16.1	469.3±41.5	436.5±17.7	497.0 ±48.0§	469.4±47.3	413.7±26.8	406.8±20.2
S5	-	-	-	514.0±17.3	-	-	-	-
REC	-	350.3±44.7	508.6±115.5#	721.2±64.7#	72.3±9.3	657.3±69.3*#	849.0±54.2#	1270.3±58.1*#

for all groups at the end transport (range of groups: 2.18-3.28 / 2.85-3.14 mg L^{-1}FW/ SW) compared to before transport (0.03/ 0.04mg L^{-1}FW/ SW). The ammonia excretion levels did not show significant difference between groups or salinities (range of groups: 13.92-19.48 / 26.30-27.81 mg kg^{-1} h^{-1} FW/ SW)

Discussion

Analysis of the major constituents of the EO-Y and EO-O of *Nectandra megapotamica* indicated almost the same qualitative composition. Both EOs presented bicyclogermacrene, α- and β-pinene and germacrene D as major compounds, but the concentration of the substances varies according to the development stage of the leaves. For the EO of the same species growing in São Paulo state (Brazil), literature describes α-bisabolol as major constituent (Romoff *et al.*, 2010).

The major compounds of EO of *N. megapotamica* can play an important role in the observed pharmacological effect. Both α- and β-pinene have been reported as sedatives (Mercier *et al.*, 2009) and analgesics (Erazo *et al.*, 2006). Kasanen *et al*. (1998) also reported that all pinenes induced sedation followed by signs of anesthesia in mice. Moreover, α-pinene is reported as positive modulator of $GABA_A$ receptors (Aoshima & Hamamoto, 1999). These receptors are recognized as important targets for modulation by sedative, anxiolytic and general anesthetic agents (Franks & Lieb, 1994). Del-Vechio-Vieira *et al*. (2009) described the analgesic activity of EO from leaves of *Ageratum fastigiatum* in mice, whose main component was germacrene D. Studies regarding this activity related to bicyclogermacrene were not found in literature. To the best of our knowledge this is the first study that describes the anesthetic activity of this EO.

Results of the anesthetic activity have shown that the concentration required of both EOs of *N. megapotamica* to induce mild sedation in fat snook adapted to both salinities was 30 μL L^{-1}. At concentrations of 150 μL L^{-1} or higher, the induction of deep anesthesia (stage 4) was achieved in 5.6-8.0 min. Although ethanol has presented anesthetic effect at the highest concentration used to dilute the EO, this effect was weaker than the effect observed for the oil and was transient. The recovery time of anesthesia induced for this concentration varied between 5.5-11.0 min. According to Marking & Meyer (1985) an ideal fish anesthetic should induce anesthesia in less than three min, and the recovery should occur within five min. Studies with EOs of different plant species have shown variations in efficacy and safety, depending on the chemical composition and the fish species tested. Menthol, the main component of EO of plants from genus *Mentha*, can induce

Table 3. Plasma glucose, lactate, $Na^+$and K^+ levels in fat snook *Centropomus parallelus* acclimated to fresh- or seawater and exposed for 10 min to the essential oil from old leaves of *Nectandra megapotamica* (30 and 300 μL L^{-1}). BC: basal control; AC: aquarium control. Data presented as means ± SEM (n = 6). Different lower case letters indicate significant difference between groups in the same salinity, while capital letters indicate significant difference between salinities in the same group.

	Freshwater		Seawater	
Groups	Glucose (mg dL^{-1})	Lactate (mg dL^{-1})	Glucose (mg dL^{-1})	Lactate (mg dL^{-1})
BC	$45.16±9.25^{aA}$	$26.40±3.05^{aA}$	$45.80±11.80^{aA}$	$30.37±0.43^{aA}$
AC	$53.33±11.57^{aA}$	$43.46±1.91^{aA}$	$116.33±12.87^{bB}$	$75.88±6.32^{bB}$
30	$68.33±17.92^{aA}$	$45.75±5.62^{aA}$	$125.33±11.16^{bB}$	$62.55±7.16^{bA}$
300	$109.85±23.54^{bA}$	$53.94±6.39^{bA}$	$159.00±13.83^{bB}$	$67.06±9.41^{bA}$
	Na^+ (mmol)	K^+ (mmol)	Na^+ (mmol)	K^+ (mmol)
BC	$175.00±8.21^{aA}$	$3.52±0.45^{aA}$	$226.20±13.46^{bB}$	$3.80±0.70^{aA}$
AC	$165.25±11.15^{aA}$	$3.08±0.66^{aA}$	$220.80±4.57^{bB}$	$2.12±0.23^{aA}$
30	$161.60±12.49^{aA}$	$4.02±0.65^{aA}$	$197.80±17.71^{bB}$	$2.58±0.50^{aA}$
300	$191.75±9.89^{aA}$	$3.48±0.81^{aA}$	$221.60±5.75^{bA}$	$2.04±0.40^{aA}$

Table 4. Tissue water content (%) in fat snook *Centropomus parallelus*, under control conditions (BC: basal control; AC: aquarium control) or submitted to the essential oil from old leaves of *Nectandra megapotamica* (30 and 300 μL L^{-1}). Data presented as means ± SEM (n = 6). No significant difference between groups was observed (P> 0.05).

Groups	tissue water content %			
	Freshwater		Seawater	
	gills	muscle	Gills	muscle
BC	73.30±0.82	79.94±0.53	70.40±2.86	76.55±0.75
AC	72.25±0.63	79.48±0.31	72.33±0.96	77.65±0.73
30	74.05±1.60	80.77±0.42	70.70±1.94	78.60±0.61
300	73.00±1.18	80.17±0.84	68.50±0.80	76.36±0.60

deep anesthesia in *Colossoma macropomum* at 100 mg L^{-1} in 4.39 min, whereas recovery occurred in 5.04 min (Façanha and Gomes, 2005) and for the same concentration in *Piaractus mesopotamicus* induction and recovery time were less than two min (Gonçalves *et al.*, 2008). The EO of *Lippia alba* was effective to induce stage 4 in silver catfish (*Rhamdia quelen*) at 300 mg L^{-1} in 3.8 min and recovery in 6.4 min (Cunha *et al.*, 2010a). The effect of eugenol (major component of clove oil), varies according to the species of fish, but 30-50 mg L^{-1} induced deep anesthesia within 2-8 min in red pacu, *Piaractus brachypomus* (Sladky *et al.*, 2001), silver catfish, *Rhamdia quelen* (Cunha *et al.*, 2010b), and goldfish, *Carassius auratus* (Abdolazizi *et al.*, 2011). The recovery time ranged from 1-10 min. In general, the recovery time of the EO-O was higher than EO-Y, and this may be due to the difference in the concentrations of the components of EOs.

The efficacy of anesthetic agents can be affected by biological (species, age, size, and sex) and environmental (salinity, pH, oxygen level, and water temperature) factors (Ross & Ross, 2008). In this study, we compared the anesthetic activity of EO of *N. megapotamica* at different salinities through the times of induction and recovery of anesthesia and, in general terms, there was a shorter induction and recovery times in FW- adapted fat snook. Ghazilou *et al.* (2010) observed that Caspian salmon [*Salmo trutta caspius* (= *Salmo caspius*)] exposed to clove oil at different salinity levels showed an increase in the induction time with increasing salinity. According to Ross & Ross (2008), due to the buffering capacity of SW and its ionic constituents, the effects of some drugs may be modified in SW.

Euryhaline fish maintain plasma glucose and lactate levels constant within their optimum salinity range, and alterations are observed when these fish are exposed to extreme salinities (Arjona *et al.*, 2007; Herrera *et al.*, 2009). No significant changes were observed in these parameters in fat snooks, indicating that the acclimation to FW and SW did not require higher energy demand. Some anesthetics can inhibit stress by acting on the hypothalamo-pituitary-interrenal (HPI) axis reducing or blocking its activity (Oslen *et al.*, 1995). Failure to suppress the activation of the HPI axis during stress results in rapid release of catecholamines and consequent increase in circulating levels of glucose and lactate (Barton, 2002). Results observed in the present study showed that the EO-O of *N. megapotamica* did

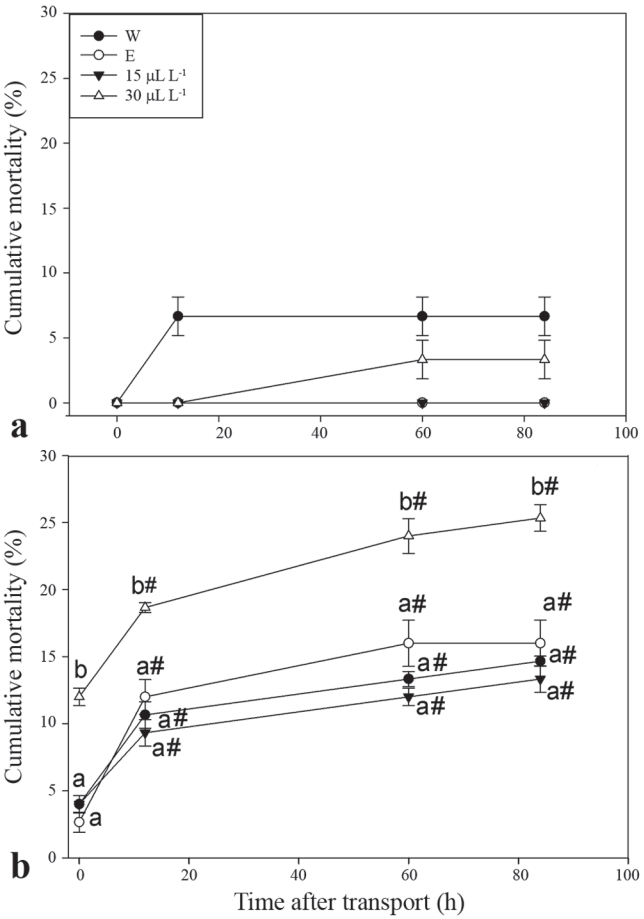

Fig. 1. Mortality after transport of fat snook *Centropomus parallelus* in plastic bags with essential oil from old leaves of *Nectandra megapotamica* (15 or 30 μL L^{-1}) or ethanol (E) added to the water. W: control with only water. Data presented as means ± SEM (n = 3). **a**, freshwater - no significant difference between groups or times was observed and the treatments E and 15μL L^{-1} are superimposed on the first line ; **b**, seawater - values with different superscripts are significantly different (P < 0.05). # Significant difference from arrival (0 h).

not change plasma glucose levels in SW-adapted fat snooks. However, in fish maintained in FW the exposure to the higher concentration tested of this EO apparently induced stress because plasma glucose and lactate levels were significantly higher than in fish from other treatments. Elevated levels of glucose were also observed after the induction of anesthesia with eugenol in common carp (Velisek *et al.*, 2005), and rock bream, *Oplegnathus fasciatus* (Park *et al.*, 2009). Rise of plasma lactate concentration was also reported with the use of eugenol in Nile tilapia, *Oreochromis niloticus* (Deriggi *et al.*, 2006).

According to Lee *et al.*, 2009, among the secondary responses to stress, osmoregulatory disturbances may be observed and evidenced by changes in water content of tissues and plasma ions. Muscle and gill water content can be used as a physiological index to evaluate the balance of water content in

fish. Our results showed no significant change in water content of fat snook muscle and gills or Na^+ and K^+ plasma levels between anesthetized fish and controls in the same salinity. Plasma ion levels of fat snook were within the expected range for tropical fish (Becker *et al.*, 2011). SW-adapted fish showed a higher Na^+ plasma levels than FW-adapted ones, and this was expected because SW acclimation increased osmolality in Mozambique tilapia (*Oreochromis mossambicus*) (Fiess *et al.*, 2007) and plasma level of Na^+ and Cl^- in European flounder (*Platichthys flesus*) (Lundgreen *et al.*, 2008), *Micropogonias furnieri* and *Genidens barbus* (Becker *et al.*, 2011) compared to FW-adapted specimens.

Control of water quality and fish sedation during transport can be useful tools to minimize stress (Ross & Ross, 2008). In the present study, water quality at the end of transport was within acceptable values for snooks (Alvarez-Lajonchere & Tsuzuki, 2008). Unionized ammonia (NH_3) levels at the end of transport were 0.0085 and 0.016 mg L^{-1} for FW and SW, respectively, therefore much lower than toxic levels for fish (Randall & Tsui, 2002; Miron *et al.*, 2008). However, EO-O did not reduce ammonia excretion, and consequently did not prevent deterioration in water quality post-transport. In addition, mortality was not lower with the use of EO-O, on the contrary, fish transported with the higher concentration of EO in SW had significantly higher mortality than the other groups. Several studies have shown that sedation can decrease post-transport mortality (Inoue *et al*., 2005; Iversen *et al*., 2009; Pramod *et al*., 2010). However, in this study an increase of mortality after transport was detected at 30 µl/L EO-O. This could be related to the concentration and duration of exposure during transport in this study. In conclusion, the EO of *N. megapatomica* is effective at inducing mild sedation and deep anesthesia in *C. parallelus*, however, it does not prevent the stress caused by anesthesia and transport according to parameters analyzed. Further studies aiming to isolate the substances with anesthetic potential and activity tests with different fish species, as well as other stress parameters (*e.g.*, cortisol) may better elucidate the anesthetic activity of this essential oil.

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