

Effects of clove oil on the stress response of matrinxã (*Brycon cephalus*) subjected to transport.

Luís Antônio Kioshi Aoki INOUE¹; Luís Orlando B. AFONSO²; George K. IWAMA³; Gilberto MORAES^{1*}

ABSTRACT

Fish transport is one of the most stressful procedures in aquaculture facilities. The present work evaluated the stress response of matrinxã to transportation procedures, and the use of clove oil as an alternative to reduce the stress response to transport in matrinxã (*Brycon cephalus*). Clove oil solutions were tested in concentrations of 0, 1, 5 and 10 mg/L during matrinxã transportation in plastic bags, supplied with water and oxygen as the usual field procedures in Brazil. Clove oil reduced some of the physiological stress responses (plasma cortisol, glucose and ions) that we measured. The high energetic cost to matrinxã cope with the transport stress was clear by the decrease of liver glycogen after transport. Our results suggest that clove oil (5 mg/l) can mitigate the stress response in matrinxã subjected to transport.

KEY WORDS

matrinxã *Brycon cephalus*, transport, stress, clove oil.

Efeito do óleo de cravo na resposta de estresse do matrinxã (Brycon cephalus) submetido ao transporte.

RESUMO

O transporte de peixes vivos é certamente um dos principais estímulos adversos à homeostase dos peixes nas condições de criação em cativeiro. O presente trabalho mensurou o estresse do matrinxã (*Brycon cephalus*), quando submetido ao transporte em sacos plásticos, bem como avaliou os efeitos do uso do anestésico óleo de cravo nessa etapa do manejo. Foram testadas as concentrações de 0, 1, 5 e 10 mg/L de óleo de cravo em bolsas plásticas preenchidas com água e oxigênio, de acordo com as práticas comumente utilizadas no Brasil. O óleo de cravo reduziu algumas das principais respostas ao estresse (cortisol, glicose plasmática e íons) mensuradas. O alto gasto de energia para o matrinxã tolerar o transporte foi evidenciado pela diminuição dos valores de glicogênio hepático. Os resultados sugerem que o óleo de cravo em concentração de 5 mg/L pode atenuar as principais respostas de estresse do matrinxã durante o transporte.

PALAVRAS-CHAVE

matrinxã *Brycon cephalus*, transporte, estresse, óleo de cravo.

INTRODUCTION

Matrinxã *Brycon cephalus* (Gunther, 1869) is a warm-water teleost fish from the Amazon basin that has been intensively reared in South America due to its favorable aquaculture characteristics, such as growth and feed conversion ratios (Carneiro & Urbinati, 2001). However, mortality in matrinxã has been reported in many fish farms, and it is usually associated with acute stimulus as handling and transport (Kubitza, 2003). These procedures are necessary, although they elicit many physiological and biochemical responses, collectively known as the stress responses (Iwama *et al.*, 1997).

Anesthetics are currently used to minimize the stress associated with aquaculture procedures. The choice of a particular anesthetic depends on the availability, cost, safety to fish and humans (Iwama & Ackerman, 1994). Several anesthetics sedate fish facilitating several stressful procedures (Iversen *et al.*, 2003; Pirhonen & Schreck, 2003). Clove oil is one of the alternatives. Clove oil is extracted from clove tree, and the main component is eugenol (4-allyl-2-methoxy-yphenol), which is traditionally used in dentistry as a component of several odontological products. In the past, clove oil was used as anti-

¹Departamento de Genética e Evolução (DGE), Universidade Federal de São Carlos (UFSCar). CP 676, CEP 13565-905, São Carlos, SP, Brasil.

* Corresponding author. Telephone +55-16-3351 8376, Fax +55-16-3351 8377, email: gil@power.ufscar.br

²Institute for Marine Biosciences (IMB), National Research Council of Canada (NRC/CNRC). 1411 Oxford St. B3H 2Z1. Halifax, NS, Canada.

³Arcadia University, Department of Biology, B3H 4P4. Wolfville, NS, Canada.

septic and local analgesic for toothache (Soto & Burhanuddin, 1995). The FDA (US Food and Drugs Administration) considers clove oil as a safe substance (Anderson *et al.*, 1997), and it has been used as a food additive. Clove oil is approved for use in aquaculture facilities in Australia, New Zealand, and other Oceania countries with no withdrawal period for human consumption and release of the fishes in the environment (Kildea *et al.*, 2004). However, in North America the FDA does not approve the use of clove oil for aquaculture purposes (Sladky *et al.*, 2001)

Researchers have investigated the effects of clove oil in fishes (Soto & Burhanuddin, 1995; Keene *et al.*, 1998; Cho & Heath, 2000; Sladky *et al.*, 2001; Tort *et al.*, 2002; Woody *et al.*, 2002; Small, 2004). A study demonstrating the efficacy of clove oil in matrinxá (Inoue *et al.*, 2003), but this work only determined the induction and recovery times to anesthesia.

The use of a natural anesthetic in matrinxá transportation would be an alternative to reduce the stress response that is certainly unavoidable, but it claims for new strategies to ameliorate this species management. Therefore, in this study we examined the effects of clove oil on the stress response of matrinxá subject to transportation.

MATERIAL AND METHODS

Fish

Juveniles matrinxá were purchased from a commercial fish farm and maintained in closed fiberglass tanks with re-circulated water for 4 months. The tanks were 2-m³ capacity and the water quality parameters were: 25.7 ± 0.9 °C, oxygen 5.66 ± 0.07 mg/L, conductivity 74.3 ± 4.8 mS.cm and pH 7.0. Fish were fed with commercial pellets (30 % protein), twice a day to satiety. Fish average weight and length were 80.1 ± 18.4 g and 18.1 ± 1.4 cm, respectively.

Experiment I

A preliminary study was done to determine whether clove oil would affect cortisol response in matrinxá subjected to transport. Clove oil was obtained from the local market as pharmaceutical grade product, and an alcoholic solution was prepared to evade the clove oil hydrophobic traits. One part of clove oil was diluted in 20 parts of 100 % ethanol. Clove oil density was approximately 1000 mg/mL (National Toxicology Program, 2002), and final clove oil alcoholic solution was 50 mg/mL.

Clear plastic bags (50 x 85 cm) were filled with water (10-L), and the respective amount of clove oil (previously diluted in alcohol) was added to obtain the following final concentrations: 0, 1, 5, and 10 mg/L. Forty matrinxá were netted from the holding tanks and transferred to four plastic bags (equally distributed) corresponding to approximately 80 g/L, which is the density usually used to transport matrinxá (Urbinati *et al.*, 2004). Pure oxygen was supplied to the bags, which were sealed, placed in a transport truck and driven around for a

period of 4 hours (from 10 am to 2 pm). At the end of the transportation, all the fish from each bag were sampled for posterior plasma cortisol and glucose analysis. Two control groups were performed; one at the beginning of the transportation and the other at the end of it. In both cases, ten fish were sampled from the holding tanks for the same analysis and were called CB and CA respectively.

Experiment II

The results from experiment I revealed that clove oil in concentration of 5 mg/L could be suitable to attenuate the cortisol and glucose responses. Therefore, in experiment II, we tested this dose, and we analyzed the main aspects of the primary and secondary stress responses (plasma cortisol, glucose, total ammonia, lactate, protein and ions and liver glycogen). In this study two groups (with 3 replicates and 10 fish in each bag) were compared: control (no anesthetic) and clove oil (5 mg/L). Additionally, before transferring the fish to the bags, 10 animals from the holding tank were sampled (Unstressed group). Experimental conditions were the same as described for experiment I.

At the end and the beginning of transport period, the water temperature, dissolved oxygen and pH were electrometrically measured. Water samples were also collected and frozen for posterior analyses of total ammonia by nesslerization (Gentzkow & Masen, 1942).

Fish sampling

Fish were killed by a sharp blow to the head. Weight (g) and length (cm) were recorded from each fish, and blood (0.5-1.0 mL) was collected from the caudal vein using heparinized 3 mL syringes. Blood aliquots were centrifuged at 12000 g for 3 min to collect plasma. The whole liver was quickly removed from each fish and immediately frozen in liquid nitrogen. Plasma and livers were stored at -20 °C until assays. Except for plasma cortisol (n=6), the other analyses were performed in all fish from each treatment.

Biochemical determinations

Plasma glucose levels (mg/dL) were measured in duplicate using a modified Trinder (1969) enzymatic assay available in kit form (Sigma, Mississauga, ON, Canada). The absorbance was read on a Molecular Devices SpectraMax 340PC microplate reader ([control Y] = 525 nm). Plasma cortisol levels were measured in duplicate using an enzyme-linked immunosorbent assay kit from the Neogen Corporation (Lansing, MI, USA). Cortisol was measured directly from plasma samples on a microplate reader ([control Y] = 450 nm). Plasma aliquots (100 ml) were disrupted in 1 mL TCA (20 % Tri chlor acetic acid) and centrifuged at 12000 g for 3 min. Total ammonia (Gentzkow & Masen, 1942) and lactate (Harrower & Brown, 1972) were determined according colorimetric methods. Other plasma aliquots were used for protein determinations according to Lowry *et al.* (1951). Plasma sodium (Na⁺) and potassium (K⁺)

were quantified through flame photometry (Digimed-DM61). Plasma chloride was determined according a chlorimetric method (APHA, 1980). Liver samples were also collected, and disruption was held in alkaline conditions at 100 °C. Glycogen determinations were according to Bidinotto *et al.* (1997).

Water quality analyses

Temperature and dissolved oxygen were measured using an YSI model 55 oxygen meter. pH was measured in a Oreon model 710A pH meter. Water total ammonia was determined by nesslerization by a colorimetric method (Gentzkow & Masen, 1942).

Statistical analysis

All data are represented as mean ± SEM. The data were submitted to ANOVA, and the Tukey test was used to discern differences among the means ($P < 0.05$).

RESULTS

No fish mortality was observed in the course of transportation.

Experiment I

Plasma cortisol levels were higher in all groups subjected to transportation (Table 1). The data also showed that fish transported in bags containing clove oil in the concentration of 1 mg/L presented the highest cortisol values. Fish subjected to transport in bags containing clove oil in concentration of 5 and 10 mg/L showed lower cortisol levels than fish transported in bags with no clove oil addition. However, these cortisol values were higher than both controls (CB and CA). The results of plasma glucose levels followed the same pattern of cortisol (Table 1). Fish were apparently tranquilized having reached the stage I of anesthesia (Woody *et al.*, 2002) during transport.

Experiment II

Plasma cortisol levels were significantly higher in all fish subjected to transport (Fig 1A). However, plasma cortisol levels in fish transported in bags containing clove oil (5 mg/L) were significantly lower than fish transported without anesthetic (0 mg/L).

Plasma glucose levels in the group transported without clove oil were significantly higher than control before and fish

transported in bags containing 5 mg/L of clove oil (Fig 1B). There was no significant difference in glucose levels between the unstressed fish and the group transported in clove oil solution.

Plasma lactate levels in the group transported without clove oil were significantly higher than the unstressed and fish transported in bags containing 5 mg/L of clove oil (Fig 2A). There was no significant difference in lactate levels between the unstressed and the transported fish in the bags with clove oil addition.

Plasma ammonia levels were significantly higher in all fish subjected to transport (Fig 2). Plasma ammonia levels in transported fish in bags containing clove oil (5 mg/L) were significantly lower than fish transported with no anesthetic (0 mg/L). However, fish from clove oil group had significant plasma ammonia increase when compared with the unstressed fish.

Plasma protein levels were similar in all groups (Fig. 2C). Hepatic glycogen levels were significantly lower in the groups subjected to transport, and there was no significant difference in the levels between transported fish in bags containing clove oil (5 mg/L) or not (0 mg/L) (Fig 2D).

Plasma sodium was similar in all groups (Fig 3). However, chloride and potassium levels were significantly lower in fish transported without clove oil addition (0 mg/L). There was no significant difference in the levels between the transported fish in bags containing clove oil (5 mg/L) and the unstressed groups.

Water quality parameters were the same in the unstressed fish during the experiments. During the transportation total ammonia increased, and pH decreased in all the bags (Table 2). As previously tested, clove oil addition to the water has no effect on its quality parameters.

DISCUSSION

Transport stress has been documented in several fish species. Usually the transport procedures promote changes in the physiological indicators of the stress such as cortisol, glucose, and ions (Wandelaar-Bonga, 1997). As transport is unavoidable in aquaculture, it is important to investigate strategies to minimize the physiological changes associated with this procedure. In this study, we examined the effects of clove oil (5mg/L) on a 4-h transport of juvenile matrinxá. Our results demonstrated that clove oil alleviated most of the measured aspects of the stress response when compared with transported fish with no anaesthetic.

The control group (transport without anaesthetic) showed an increase in plasma cortisol levels when compared with both

Table 1 - Effect of clove oil on the plasma cortisol (ng/mL) and glucose (mg/dL) of matrinxá submitted to transportation in plastic bags. Unstressed groups (CB-control before and CA-control after) were separately sampled before and after stressors. Different upper-script letters means significantly different at $p < 0.05$ in the rows.

Treatment	Unstressed		Clove oil solutions				Unstressed
	CB		0 ppm	1 ppm	5 ppm	10 ppm	CA
Cortisol	12.2 ± 1.8c		26.3 ± 1.2a	30.7 ± 5.2a	23.8 ± 1.7b	22.0 ± 1.5b	11.7 ± 1.2c
Glucose	95.5 ± 3.7d		163.3 ± 3.3b	180.0 ± 3.4a	133.3 ± 2.5c	155.5 ± 2.9b	95.5 ± 1.8d

unstressed (not subjected to handling or transportation) and transported fish in bags containing clove oil solution (5 mg/l). Although clove oil attenuated the cortisol response, it did not abolish it completely. In our study, fish were exposed to a 4-h transport in a low concentration of clove oil (5 mg/L). Most of the studies have exposed fish to higher concentrations of clove oil (10-140 mg/L) for shorter periods (1-30 minutes) (Iversen *et al.* 2003; Small, 2004; Woody *et al.*, 2002; Keene *et al.*, 1998). Some of these studies have shown that the cortisol response can be prevented when fish are exposed to high

doses of clove oil. For instance, Atlantic salmon did not elicit cortisol response when exposed to 20-100 mg of clove oil/l during a 30 min exposure (Iversen *et al.*, 2003). Catfish also responded in a similar way when exposed to 100 mg of clove oil/L (Small, 2004). Study with Atlantic salmon demonstrated that exposure to 10 mg/L of clove oil prevented the cortisol response only during the first 10 min of exposure. After that, plasma cortisol levels significantly increased, but the values were lower (attenuated response) than fish not exposed to clove oil (Iversen *et al.*, 2003). Although in our study we did not measure cortisol levels throughout the 4-h transport, we observed attenuation on the cortisol response in fish transported in bags containing clove oil. The mechanism of how clove oil affects the cortisol response is not known. Iversen *et al.* (2003) speculated that clove oil may block the transmission of sensory information to the hypothalamus, and therefore high concentrations of anesthetics prevent the activation of the hypothalamus-pituitary-interrenal (HPI) axis more effectively than lower concentrations. So the cortisol response may be prevented (Iversen *et al.*, 2003).

The use of clove oil prevented the usual increase in plasma glucose levels associated with fish transport. Similarly, Iversen *et al.* (2003) showed that Atlantic salmon did not increase plasma glucose levels during 30 min exposure to different concentrations of clove oil. Cortisol is thought to be one of the mediators of the increase in plasma glucose levels seen in stressful events (Barton *et al.*, 2002). It may be possible that the lack of glucose response in fish exposed to clove oil was due to the attenuation of the cortisol response by clove oil.

Fish transport in plastic bags containing clove oil prevented plasma lactate rise that is usually seen when oxygen is not available for aerobic cell metabolism (Iversen *et al.*, 2003). It usually takes place after stressful events that involve elevated muscular activity like burst swimming or severe exercise (Barton *et al.* 1998). In

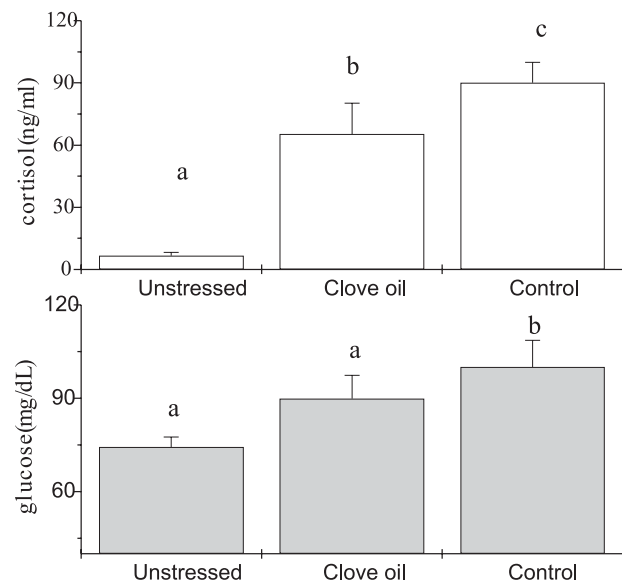


Figure 1 - Effect of clove oil on plasma cortisol and glucose of matrinxá submitted to transport. Clove oil was used in the concentration of 5 mg/L, Control group had no clove oil addition, Unstressed group were neither subjected to handling nor transport.

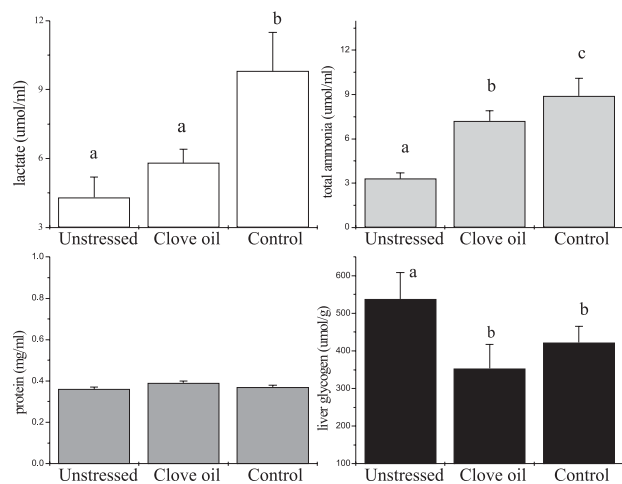


Figure 2 - Effect of clove oil on plasma lactate, total ammonia, protein and liver glycogen. Clove oil was used in the concentration of 5 mg/L, Control group had no clove oil addition, Unstressed group were neither subjected to handling nor transport.

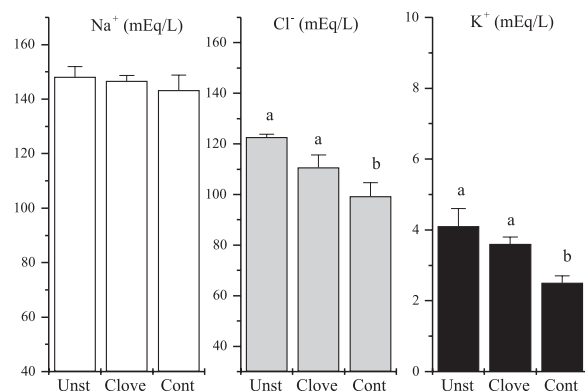


Figure 3 - Effect of clove oil on the ionic balance of matrinxá submitted to transportation in plastic bags. Clove oil was used in the concentration of 5 mg/L (Clove). Control group (Cont) had no clove oil addition. Unstressed group (Unst) were neither subjected to handling nor transport.

Table 2 - Effect of clove oil on water quality of transport in matrinxá. Fish transport was held in plastic bags (50 x 85 cm) containing 10 L of water (fish density of 80 g/L) during 4 h. Non-ionized ammonia (NH₃) were calculated from total ammonia according to the pH and temperature values. Different upper-script letters means significantly different at p<0.05 in the columns.

Transport condition	Total ammonia NH ₃ + NH ₄ +mg/L	Unionized ammonia NH ₃ mg/L	pH	Temperature °C	Oxygenmg/L
Unstressed	0.87 ± 0.12b	0.05 ± 0.01	7.8 ± 0.4a	24.1 ± 0.1	5.03 ± 0.2
Clove oil	7.97 ± 0.52a	0.04 ± 0.01	6.8 ± 0.06b	24.1 ± 0.1	19.31 ± 0.52a
Control	8.07 ± 1.05a	0.04 ± 0.02	6.7 ± 0.2b	24.1 ± 0.1	16.89 ± 1.25b

this study, the lack of lactate response was probably due to the apparent lower muscular activity in fish exposed to clove oil. However, the decreased muscular activity, likely did not interfere with the respiration and ventilation ratios. Decrease on these parameters are frequently associated with anesthetics used in high concentration, which causes the decreased availability of oxygen to the cells, and therefore eliciting the increase of plasma lactate, a by-product of the anaerobic metabolism (Barton *et al.*, 2002). Atlantic salmon only showed slight increase in plasma lactate levels 30 min after exposure to 10 mg of clove oil/L, and the time of increase was related to the higher stages of anesthesia reached at that time (3a: total loss of equilibrium – fish usually turn over but retain swimming ability) (Iversen *et al.*, 2003).

Our results indicated that transporting fish with or without clove oil increased plasma ammonia levels. However, the increase was attenuated by clove oil. In fish, nitrogen is excreted as ammonia mainly via diffusion of unionized ammonia (NH₃) across the gills (Wright, 1993). It is known that high levels of ammonia in the water will likely reduce the NH₃ gradient between fish blood and water. It is possible that the increased total ammonia levels in the water (Table 2) impaired the ammonia excretion, and consequently increased plasma ammonia levels.

Plasma protein levels were similar in all treatments. Although plasma protein level is not a very sensitive stress indicator, it usually shows some internal water unbalance and possibly osmoregulatory problems (Barton *et al.*, 2002).

Juvenile matrinxá subjected to a 4-h transport showed marked liver glycogen depletions. Decreased glycogen reserves are usually associated with exhaustive activities. It is difficult to explain why the fish exposed to the anesthetic (which did have their movements decreased) showed the same depletion of hepatic glycogen store. This should indicate that the demand of energy to cope with the anesthetic processes was similar among handled fish and that handled plus anesthetic. Such assumption is supported by the similar pattern of plasma ammonia increase observed in the both group of fish. The glycogen depletion in transported fish without anesthetic makes sense, since the animals had increased muscular activity. Furthermore, these animals catabolized glucose through anaerobic strategy since their plasma lactate levels increased drastically. The levels of liver glycogen stores allow us to suppose that the presence of clove oil, in spite of reducing the levels of plasma cortisol, is still working as significant stressor. Moreover, the content of liver glycogen is a good parameter to infer the stress response since it be associated to other metabolites.

Our study also showed clove oil prevented the usual ions concentrations decreases associated with transportation. Several studies in freshwater fish have demonstrated decreased plasma chloride levels in animals subjected to transport (Barton *et al.*, 2002; Forsberg *et al.*, 2001; Mazik *et al.*, 1991). A study with matrinxá showed that addition of marine salt (NaCl) to the water can also minimize the loss of ions during transportation (Carneiro & Urbinati, 2001). This, however, may not be valid for all species, since in walleyes (*Sander vitreus*) addition of salt (0.5%) did not prevent the decrease in plasma chloride (Carmichael *et al.*, 2001).

It is believed that the use of anesthetics in fish transport may reduce the fish activity and the ammonia excretion through the gills. Consequently, clove oil would provide better water quality for transport, and larger amounts of fish could be transported in the same container (Kubitza, 1998, Kubitza, 2003). However, the results of the water analysis in this study demonstrated that during transport the water quality deteriorated in all treatments, and the anesthetic addition did not attenuate the water deterioration as expected (Table 2). Water pH decreased after the 4-h transport in the plastic bags probably as a result of CO₂ accumulation. Bohr Effect is evident in the water of fish transport in Brazil because the waters are softly acidic (Esteves, 1988). The pH influences the toxicity of several substances including total ammonia, which is present in the water as two forms: un-ionized (NH₃ – toxic to the fish, it diffuses easily across the gills) and ionized (NH₄⁺). At low pH, un-ionized ammonia represents a small portion of the total ammonia (Boyd, 1982). Although in our study the levels of total ammonia increased during transportation (Table 2), the levels of un-ionized ammonia were low (due to the reduction in the pH) and probably were not toxic to the fish. Furthermore, water temperature was constant avoiding another variant of ammonia toxicity. We also had high dissolved oxygen levels (Table 2), which was due to the fact we saturated the bags with pure oxygen as usually fish farmers do in field conditions. However, the over oxygen saturation condition in the bags apparently did not have any deleterious effect on matrinxá.

In summary, this study suggests that the use of clove oil attenuated the stress response in matrinxá during transportation. Overall, from the ten physiological parameters we measured, four responses were prevented (glucose, lactate, chloride, and potassium), two were attenuated (plasma cortisol and ammonia), and two were not altered (sodium and protein). However, clove oil did not prevent depletion in hepatic

glycogen levels. Further studies are required to understand the physiological responses of matrinxá exposed to clove oil during other periods of transport and recoveries. Assessments of clove oil for aquaculture purposes have to be encouraged because this natural anesthetic is becoming more evident as a safe and low-cost alternative.

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