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Physiological changes in juvenile tambaquis (*Colossoma macropomum*) transported using essential oil of *Ocimum gratissimum*

Alterações fisiológicas em juvenis de tambaqui transportados em solução de óleo essencial de Ocimum gratissimum

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Abstract: Essential oils may be used as natural anesthetic for fish in several farming and laboratory procedures. They are considered safe for both the fish and the environment. In this way, studies that evaluate essential oils as fish stress reducers are necessary to confirm such recommendations. Transportation is one of the most stressful fish farming practices, since fish are severely disturbed. Stress among fish may induce undesired consequences such as diseases and mortality. This study evaluated the effect of *Ocimum gratissimum* essential oil on the physiological parameters of stress in *Colossoma macropomum* subjected to transportation. Fish were transported in plastic bags under different concentrations of *O. gratissimum* essential oil (0, 5, 10, 15 and 20 mg L^{-1}) for 4 h. Blood samples were collected before and after transportation. Glucose and ammonia levels increased and lactate levels decreased after transportation with *O. gratissimum* essential oil in the water. The total plasmatic protein and hepatic glycogen levels presented great variations, while hematological parameters did not show any difference between treatments. The fish recovered from transportation stress after 24 h. The concentrations of *O. gratissimum* essential oil evaluated here were not efficient in mitigating the stress responses of *C. macropomum*. Additional studies are needed to evaluate effective concentrations of *O. gratissimum* that would reduce stress responses in transportation, along with their associations with other products and procedures.

Keywords: Clove basil. Fish. Management. Stress. Hematology.

Resumo: Óleos essenciais podem ser usados como anestésicos naturais para peixes em diversos procedimentos laboratoriais ou de rotina da piscicultura. Vários são considerados seguros tanto para os peixes como para o ambiente. Entretanto, os seus efeitos como redutores de estresse em peixes necessitam ser avaliados. O transporte é uma das práticas de manejo da piscicultura mais estressantes. O estresse nos peixes pode induzir a consequências indesejáveis como manifestações de doenças e mortalidades. Este estudo avaliou os efeitos do óleo essencial de *Ocimium gratissimum* em parâmetros fisiológicos de estresse em *Colossoma macropomum* submetidos ao transporte. Os peixes foram transportados em sacos plásticos sob diferentes concentrações do óleo essencial (0, 5, 10, 15 e 20 mg L^{-1}) por 4 h. Amostras de sangue foram coletadas antes e depois do transporte. As concentrações de glicose e amônia plasmáticas aumentaram e o lactato diminuiu depois do transporte com o óleo essencial diluído na água. Os níveis de proteína plasmática e glicogênio hepático apresentaram variações muito amplas. Os parâmetros sanguíneos não mostraram diferenças entre os tratamentos. Os peixes apresentaram-se recuperados 24 h após o transporte. As concentrações avaliadas do óleo essencial de *O. gratissimum* não foram eficientes como mitigadores de estresse em tambaqui submetido ao transporte. Estudos adicionais são necessários para avaliações de outras concentrações, bem como a associação com outros produtos e procedimentos.

Palavras chave: Alfavaca cravo. Peixe. Manejo. Estresse. Hematologia.

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Introduction

Transportation is one of the most stressful field practices of fish farming (Gomes *et al*., 2003). However, it is indispensable for putting this activity into operation, such as for commercialization of fingerlings and juveniles, or during storage of live fish in depuration tanks, in preparation for industrial slaughter and processing (Gressler *et al*., 2015; Sampaio & Freire, 2016, Stringhetta *et al.,* 2017).

The practices involved in fish transportation include handling and crowding, along with changes in the water quality. These induce stress responses that can affect the productive performance and resistance of fish to diseases (Brandão *et al.,* 2006; Sampaio & Freire, 2016).So several procedures and products have been tested to improve this important operation within fish farming (Gressler *et al*., 2017; Sampaio & Freire, 2016, Stringhetta *et al.,* 2017).

The essential oil of *Ocimum gratissimum* (clove basil), which includes eugenol among its main constituents, has been studied as a natural anesthetic for tropical fish (Silva *et al.,* 2015; Boijink *et al*., 2016; Ribeiro *et al*., 2016). This plant is available in the tropical regions (Silva *et al.* 2015; Boijink *et al*. 2016; Ribeiro *et al.* 2016). Characteristics immunostimulatory (Brum *et al*., 2017) and antiparasitic effects (Boijink *et al*., 2016) are described. Further studies on the use of this essential oil for reduction of stress responses and fish mortality are necessary, especially in relation to fish management practices such as protocols for transporting fish in bags (closed systems). The commercial fish species can respond differently to anesthetic concentrations. Or even an essential oil may be recommended for one fish species and not for another.

Tambaquis (*Colossoma macropomum*) and hybrids resulting from crossbreeding with the pacu (*Piaractus mesopotamicus*) or pirapitinga (*Piaractus brachypomus*) are the native fish most produced in tropical regions of Brazil (IBGE, 2016). They are hardy fish that are tolerant to the extreme conditions of these regions of high temperatures and low concentrations of oxygen in the water. They also present desirable

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zootechnical indexes for growth and feed conversion in commercial farms (Valladão *et al*., 2018). Specifically regarding tambaquis, no reports on the impact of the combination of sedation and transportation on stress responses in tambaquis, using the essential oil of *O. gratissimum*, have been published. This oil would seem to be an important alternative as a natural anesthetic, since commercial anesthetics such as tricaine methanesulfonate (MS-222) are not effective in minimizing the responses to secondary or oxidative stress after transporting tambaquis (Barbas *et al*., 2017).

The objective of this study was to evaluate the effect of sedation using essential oil of *Ocimum gratissimum* on the physiological stress parameters in juvenile tambaquis subjected to transportation in plastic bags.

Material and Methods

Plants

Specimens of *Ocimum gratissimum* (clove basil) were cultivated in the medicinal plants sector of Embrapa Western Amazon in Manaus, AM (2°53'35.73" S; 59°58'23.36" W). The aerial parts were cut and air dried. Exsiccate were deposited in the Embrapa Eastern Amazon herbarium in Belém (191735), under genetic patrimony accession protocol number AB13781. The essential oil was obtained through hydrodistillation in Clevenger apparatus. The yield of essential oil was 1.5% (mass/volume), calculated according to the weight of the dry parts of the plants. Samples of the essential oil were collected for chemical characterization by means of performed by gas chromatography coupled to mass spectometry (GC-MS) (Table 1).

Table 1. Chemical composition (%) of *Ocimum gratissimum* essential oil.

Components	***O. gratissimum***	**LRI_{lit}**	**LRI_{calc}**
sabinense	0.7	969	975
β-pinene	2.8	974	976
Myrcene	0.7	988	988
1,8-cineole	28.2	1026	1030
(*Z*)-ocimene	3.7	1032	1038
Linalool	1.3	1095	1100
δ-terpineol	0.4	1162	1166
terpinen-4-ol	0.4	1174	1178
α-terpineol	1.1	1186	1190
Eugenol	43.3	1357	1357
β-bourbonene	0.9	1387	1381
β-elemene	0.8	1389	1389
(*E*)-caryophyllene	3.7	1417	1415
α-humulene	0.6	1452	1450
γ-muurolene	0.9	1478	1477
β-selinene	5.5	1489	1482
α-selinene	1.7	1498	1496
7-*epi*-α-selinene	0.4	1520	1513
Total identified	**97.1**		

Retention indices in DB-5MS. LRI_{calc}: Calculated linear retention indices. LRI_{lit}: Linear retention indices.

Fish

Juvenile tambaquis were purchased from a commercial farm in the municipality of Rio Preto da Eva, AM, and were transported to and stocked in an earthen pond of approximately 1000 m^2 at the facilities of Embrapa Western Amazon, Manaus, AM. They were fed twice daily with extruded commercial feed containing 32% crude protein until reaching 135.48 ± 36.62 g. At this time, 300 fish were transferred to a tank of volume 3 m^3. Over the next 20 days, the fish were acclimatized to the experimental conditions through maintenance of continuous water flow. The water quality variables were monitored and were maintained as follows: temperature 29.0 ± 1.7 °C; pH 7.14 ± 0.8; oxygen 6.6 ± 2.3 mg L^{-1}; hardness 8.3 ± 1.2 mg L^{-1}; alkalinity 12.7 ± 1.8 mg L^{-1}; and ammonia 0.03 ± 0.01 mg L^{-1}.

Experimental design

One day before the experimental procedures, the feeding of the fish was suspended. The experiment started with sampling of 15 fish for initial collection of biological material (blood and tissues). The fish were then distributed into 15 plastic bags (15 fish per bag) of 50 x 85 cm, filled with 20 L of water and the respective aliquots of alcoholic solution of essential oil of *O. gratissimum* (diluted 1:10), at concentrations of 0, 5, 10, 15 and 20 mg L^{-1} in triplicates. The bags were inflated with oxygen and were then sealed. Transportation was simulated by carrying the bags in a truck that ran on a highway for 4 h.

After this experimental transportation, all the containers were opened and three fish per bag were sampled for biological material collection (nine fish per treatment). The remaining fish in each of the packages were released into 15 fiberglass boxes with a capacity of 1.000 L each, with water supplied through a recirculation system, to observe their recovery from stress. For this, three fish from each box were quickly caught for collection of biological material 24 h and 48 h after transportation. The other fish remained in the 1.000 L boxes and were fed for another week. Afterwards, the fish were released and were kept in a fish pond.

Biological material and analyses

The fish were anesthetized for blood collection (100 mg L^{-1} of benzocaine) and then euthanized in accordance with the guidelines from CONCEA (2018), to obtain weight and length measurements and collect biological material. This study was developed accordingly to the ethics committee CEUA nº 03/2017 Embrapa Western Amazon. Blood collection was performed by means of caudal vein puncture using heparinized syringes (5,000 IU). The hematological parameters determined were hematocrit, hemoglobin concentration and erythrocytes count (Ranzani-Paiva *et al.,* 2013). Plasma aliquots were separated for determinations of the following biochemical parameters: glucose, lactate and total protein using commercial kits (Labtest), and total ammonia was performed as described by Gentzkow and Mazen (1942). Liver samples were collected for glycogen determination (Bidinotto *et al*., 1997).

Statistical analyses

The treatments with *O. gratissimum* were compared between the different data collection times (before transportation, shortly after transportation and at recovery times of 24 h and 48 h. The data were subjected to analysis of variance and, in the event of significant difference between the groups, the Tukey test was used to compare the means. The confidence level of $P < 0.05$ was accepted.

Results

No mortality was observed during any of the stages of this study (acclimatization, transportation and stress recovery of fish). After transportation, the plasma glucose concentration increased in relation to the initial data collection. There was no effect regarding reduction of this stress indicator through action of *O. gratissimum* essential oil. The plasma glucose valuesshowed recovery 24 h and 48 h after transportation (Figure 1A). The plasma lactate values decreased in the juvenile tambaquis during transportation, with lower plasma lactate values in the fish transported under the influence of *O. gratissimum* essential oil. The plasma lactate values were observed to have returned to levels close to those seen at the beginning of the experiment at the recovery times of 24 h and 48 h (Figure 1B). Plasma ammonia increased in all the fish subjected to transportation, and the increases were even greater in the fish that were transported under the influence of *O. gratissimum* essential oil. Twenty-four hours and 48 h after transportation, the plasma ammonia values were observed to have returned to close to those seen at the beginning of the experiment (Figure 1C).

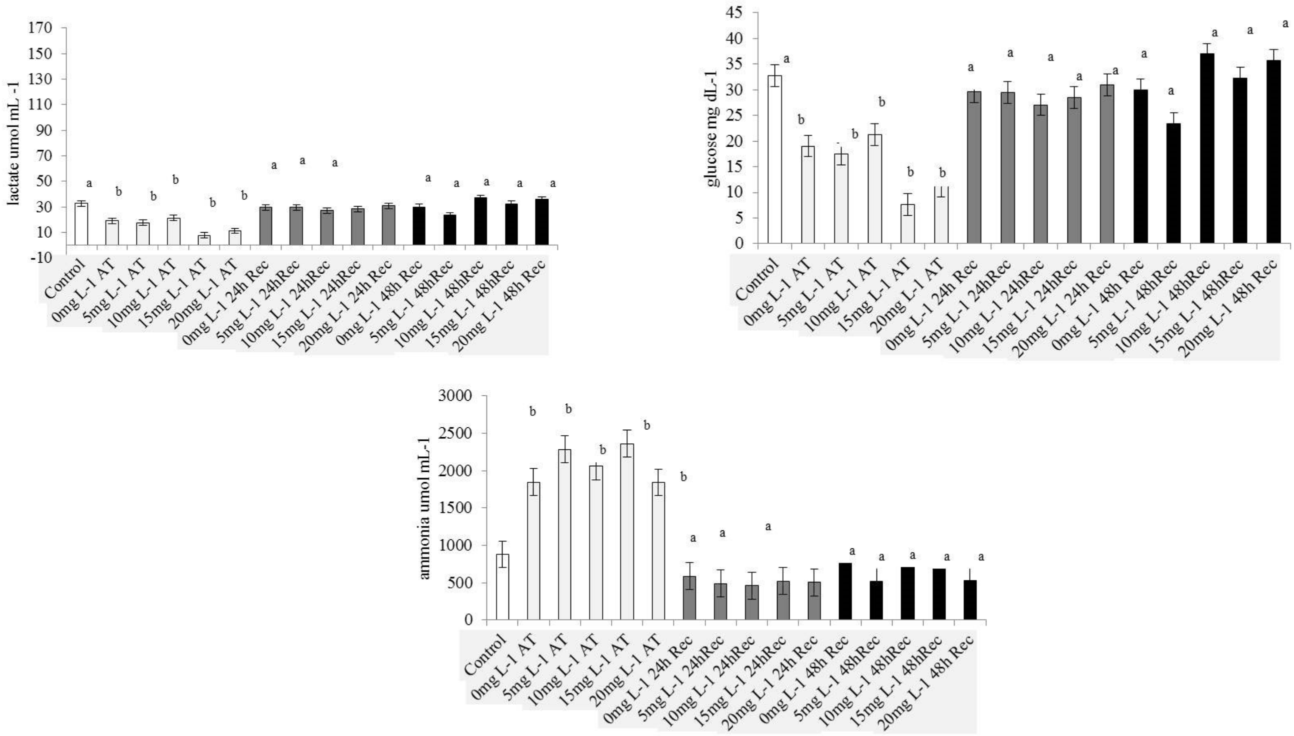

Figure 1. Biochemical parameters of *Colossoma macropomum* after transportation under the influence of *Ocimum gratissimum* essential oil. Blood samples were taken from the fish before and after transportation. The fish were allowed to recover for 24 h and 48 h. Different letters indicate significant differences among treatments Tukey test (P < 0.05).

The values of total plasma protein (4 ± 2 mg dL^{-1}) and hepatic glycogen (1506 ± 798 µmol g^{-1}) presented wide variations, and it was not possible to detect the tambaquis' response to transportation stress by means of these physiological indicators, or the possible mitigator effect of *O. gratissimum* essential oil. The blood parameters of hematocrit (32.1 ± 4.7%), hemoglobin (9.4 ± 1.6 g dL^{-1}) and erythrocyte count (1.7 ± 0.7 106 mm^{-3}) did not show any significant differences (Table 2).

Table 2. Blood of juvenile tambaquis submitted to transport stress under clove basil essential oil (O. gratissimum) influence.

	Before transport		
Treatment	Hematocrit %	Hemoglobin g dL^{-1}	RBC millions uL^{-1}
Unstressed	32.1 ± 3.29	10.29 ± 0.86	1.44 ± 0.36
	Transport		
Treatment	Hematocrit (%)	Hemoglobin (g dL^{-1})	RBC millions uL^{-1}
0 mg L^{-1}	31.22 ± 3.72	9.09 ± 1.10	1.41 ± 0.62
5 mg L^{-1}	34.22 ± 2.85	9.74 ± 0.92	1.43 ± 0.18
10 mg L^{-1}	33.38 ± 2.81	8.86 ± 2.01	1.68 ± 0.59
15 mg L^{-1}	33.66 ± 2.25	8.81 ± 2.15	1.89 ± 0.43
20 mg L^{-1}	34.5 ± 1.5	9.86 ± 1.08	1.71 ± 0.66
	24 h recovery		
Treatment	Hematocrit %	Hemoglobin g dL^{-1}	RBC millions uL^{-1}
0 mg L^{-1}	35.44 ± 2.60	9.97 ± 1.03	2.04 ± 0.54
5 mg L^{-1}	32.88 ± 2.60	9.24 ± 1.96	1.59 ± 0.79
10 mg L^{-1}	34.44 ± 3.28	9.23 ± 1.37	1.66 ± 0.76
15 mg L^{-1}	32.88 ± 3.29	9.68 ± 0.97	2.42 ± 1.02
20 mg L^{-1}	31.44 ± 4.55	8.68 ± 1.95	1.74 ± 0.80
	48 h recovery		
Treatment	Hematocrit %	Hemoglobin g dL^{-1}	RBC millions uL^{-1}

0 mg L $^{-1}$	32.22 ± 2.33	9.92 ± 0.54	1.75 ± 0.68
5 mg L $^{-1}$	25.33 ± 37.8	8.73 ± 3.89	2.18 ± 0.49
10 mg L $^{-1}$	26.88 ± 7.93	8.70 ± 1.87	1.93 ± 0.56
15 mg L $^{-1}$	30.77 ± 4.40	9.18 ± 1.10	1.37 ± 0.33
20 mg L $^{-1}$	31.00 ± 3.87	9.42 ± 0.78	2.00 ± 0.76

Values are expressed as mean ± standard deviation.

Discussion

Transportation is known to be an acute stressor agent that breaks down the balance that fish have with their environment (homeostasis), thereby initiating hormonal and metabolic responses to stress (Iwama *et al*., 2004). When transportation occurs intensely and with excessive duration, it can result in manifestation of disease throughout the fish population, with some death (Iwama *et al*., 2004; Noga, 2010). Thus, care should be taken before, during and after transportation, with teams of well-trained workers, adequate equipment and perfect sanitary conditions of the fish. Care during rearing, such as correct feeding, maintenance of water quality and use of appropriate stocking densities also influence the success of transportation (Gomes *et al*., 2003).

Different products are used to partially prevent transport stress (Gomes *et al.,* 2003; Gressler *et al*., 2017; Stringhetta *et al*., 2017). In fish transport studies, biological materials are collected for analysis before, during and after the experimental stimuli. One of the main indicators of stress in studies under field conditions is plasma glucose (Hattingh, 1976). In the present study, plasma glucose levels increased in response to transportation, but the essential oil of *O. gratissimum*, in which the major constituent is eugenol (43.3%; Table 1), did not alter this indicator of stress at the concentrations evaluated (5, 10, 15 and 20 mg L^{-1}). In a similar way to what was observed in this study Benovit *et al.* (2012), observed an increase in the glucose level of Brazilian flounders (*Paralichthys orbignyanus*) at 20 mg L^{-1} of *O. gratissimum*, which reflected the use of a combination of different stressors, i.e. anesthetic and transportation. When tambaqui and matrinxã (*Brycon amazonicus*) were anesthetized with the compound eugenol, the same responses were observed, i.e. hyperglycemia (Barbosa *et al*., 2007; Inoue *et al*., 2011). On the other hand, clove oil, in which the main component is also eugenol, decreased the glucose response in matrinxã subjected to transportation, at a concentration of 5 mg L^{-1} (Inoue *et al*., 2005). Other substances that condition transportation, such as sodium chloride, also provided lower plasma glucose values in tambaquis subjected to transportation (Gomes *et al.,* 2003). Twenty-four hours after transportation, the plasma glucose values had recovered, to close to those observed in the control treatment, which consisted of fish not subjected to stress.

During transportation of fish, it is common to record increased blood glucose levels and also reduced hepatic glycogen levels, due to mobilization of energy reserves (Pankhurst, 2011). However, the results showed that there were no accumulations of glycogen in the liver (942.34 to 2,126.47 μmol g^{-1}) after transportation of tambaqui, and no alterations to this pattern were observed 48 hours after the transportation procedures, independently of the use of *O. gratissimum* essential oil. By way of comparison, Salbego *et al.,* (2015) reported that there were higher glycogen values in the liver (605.20 μmol g^{-1}) through use of 5 μL L^{-1} of *Condalia buxifolia* extract in water used for transportation of jundiá (*Rhamdia quelen*), compared with the control.

Transportation consists of several phases such as shipping, loading of the fish in containers and the transportation itself. In general, fish move excessively in the early stages of this management practice, and the supply of energy only through aerobic metabolism may be insufficient (Barbosa *et al.,* 2007). The lactate values observed shortly after transportation indicated that there was less movement among tambaquis during transportation. In the plastic bags supplied with essential oil of *O. gratissimum* at concentrations of 15 and 20 mg L^{-1}, the muscle movements of the tambaqui were probably even smaller. More agitated species like matrinxã make intense muscle movements during transportation. Higher plasma lactate values were observed shortly after transportation of matrinxã. In addition, the use of natural anesthetics such as clove oil resulted in smaller increases in lactate due to the lower degrees of muscle movement provided by the anesthetic diluted in the transportation water (Inoue *et al*., 2005). In another study on tambaquis, use of oils from *Curcuma longa* (40 μL L^{-1}) and *Myrcia sylvatica* (10 μL L^{-1}) promoted increased plasma lactate levels in relation to controls in an open transportation system. The authors of that study recommended that these oils should be used for transporting tambaquis because they reduced lipoperoxidation (Saccol *et al*., 2016).

Nitrogen excretion in fish occurs through release of ammonia into the water. Under stressful conditions, such as transportation, this process can become altered (Carneiro & Urbinati, 2001). In the case of tambaquis transported under the influence of *O. gratissimum* essential oil, increased plasma ammonia values were observed. Also, in the plastic transportation bags containing *O. gratissimum* essential oil at a concentration of 15 mg L^{-1}, the values were high indicating that the tambaquis had some difficulty in achieving nitrogen excretion during transportation. The essential oil of *O. gratissimum* altered the exchanges between the gills and the aquatic medium more markedly during transportation. It is possible that higher concentration of the anesthetics tested in the transportation water (15 mg L^{-1}) causes decreased opercular beats in long-term baths, thereby favoring accumulation of the metabolite in the plasma temporarily (Barbosa *et al*., 2007). Twenty-four hours after the experimental stimuli, the plasma ammonia values had recovered. Other essential oils have been found to reduce total ammonia levels, such as that of *Lippia alba* (10 and 20 mg L^{-1}), which reduced this and ion loss in jundiá after transportation for 4 hours in plastic bags (Becker *et al*., 2013). Clove oil (5 mg L^{-1}) also provided decreased plasma ammonia values after transportation of matrinxã. Carneiro and Urbinati (2001) observed lower plasma ammonia values in matrinxã after transportation under the influence of salt.

During transportation, protein catabolism may be affected by osmoregulatory disorders (Baldisserotto, 2009). In the present study, the plasma protein values were not varied between treatments with essential oil of *O. gratissimum* after transportation, and no change in this pattern was observed 48 hours after the experimental procedures. In jundiá, the total protein values decreased after transportation in all treatments using the essential oil of *Myrcia sylvatica* (Saccol *et al*., 2018). On the other hand, despite the adverse stimuli imposed by sedation and transportation, the blood parameters (hematocrit, hemoglobin concentration and erythrocyte count) of tambaquis were not affected. Similar results were described by Boijink *et al*. (2016) in a study conducted using tambaquis anesthetized with *O. gratissimum* essential oil. The range of variation of the blood parameters is in agreement with what was described by Tavares-Dias (2015).

O. gratissimum essential oil has been indicated as a natural anesthetic for tambaquis (Boijink *et al*., 2016), and this effect has been attributed to eugenol and/or its synergistic association with other active compounds present in this essential oil (Silva *et al.,* 2012; Benovit *et al*., 2012; Boijink *et al*., 2015). However, in the present study, the sedation of tambaquis with *O. gratissimum* essential oil during the transportation operation was not effective in mitigating the effects of stress. Therefore, additional studies are needed to evaluate other protocols and safe concentrations of *O. gratissimum*, thus making it possible to use this essential oil in transporting live fish, along with studies on associations with other products and procedures.

Conclusion

Transport of juvenile tambaqui trigged most of the stress responses evaluated and the essential oil of *O. gratissimum*, diluted in the transportation water, did not cause an evident reduction in the physiological stress responses.

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