Full Paper

APPLICATION OF CLOVE OIL AS ANESTHETIC FOR SEA BASS (*Lates calcarifer* Bloch)

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Abstract

The objective of this study was to determine the safety and efficacy of clove oil as an anesthetic in sea bass (*Lates calcarifer)* and the potential application of clove oil as anesthetic to facilitate the sea bass fry transportation. Acute toxicity test indicated the 24-hr LC_{50} value of clove oil in sea bass fry as 30 ppm with slope function of 1.079 (1.05 to 1.107). In efficacy test, fish were exposed to 5, 10, 15, 20 and 25 ppm of clove oil for 15 minutes. At 5 ppm, clove oil caused only sedation effect (partial loss of reaction to external stimuli) while at 20 ppm, fish entered anesthesia stage (failure to respond to external stimuli) within about 3 minutes. Fish recovered from a 15-min period of exposure in 20 ppm clove oil within less than 10 minutes following removal from the anesthetic solution. There was neither mortality nor abnormal behavior of fish during 15-min exposure of clove oil as well as during 7 days post recovery from anesthesia. The potential application of clove oil as an aid in the transport of sea bass fry in plastic bag was also investigated. At 5 ppm, clove oil could reduce activities of the fish without loss of equilibrium (sedation stage) during the 4 hour simulated transport at 50 fish per 1,000 ml sea water (15 ppt). At 20 ppm, clove oil caused loss of equilibrium in fish resulting in the anesthesia stage throughout the 4 hour period. However, there was no improvement on survival rate and fish behavior with the use of clove oil during and after this 4 hour transport. Simulated transport at 50 fish per 500 ml sea water (15 ppt) for 8 hour did show better significant survival rate with additional of 5 and 20 ppm clove oil. In both short and long term transport study, clove oil did show the benefit by reducing the fish activities judging from the reduction of oxygen consumption, ammonia and carbon dioxide levels. Addition of appropriate concentration of clove oil in transport water ensured that the fish would stay calm by reducing fish activity and therefore, prevented any drastic changes of water qualities.

Key words: anesthetic, clove oil, *Lates calcarifer*, sea bass, transportation

Introduction

Aquaculture inherently involves stressing fish. Handling and transport may initiate a severe stress response in cultured fish. Transportation of live fish is a widespread practice, particularly in rural areas of developing countries and often represent-ting the only means of supplying fry to small-scale aquaculturist. Transportation is a traumatic procedure that consists of a succession of adverse stimuli including initial capture, loading into transport containers, the actual transport, unloading and stocking (Robertson *et al*., 1988). Transportation often results in mortality of fishes which may occur immediately following this severe treatment or secondarily due to osmoregulatory dysfunction or infectious diseases (Wedemeyer, 1970; Wedemeyer, 1996).

A variety of methods have been used to reduce the adverse effects of fish transport. Transport of several fish either sea water or freshwater has been facilitated by pretransport starvation, chilled shipping water and addition of

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numerous chemical in water transport. Several chemicals include oxygen-producing chemical, buffering, ammonia removal, antibiotics and anesthetics.

Anesthetics are known to lower the metabolic activity of the fishes by their depressing action on the brain. The use of anesthetics in the transport of fish can reduce the oxygen consumption and also control hyperactivity, thus preventing undue injuries. Several anesthetics have been used in the transport experiments, such as tricaine methanesulfonate, benzocaine, metomidate, 2-phenoxyethanol and quinaldine. However, those anesthetics have disadvantages for aquacultural use. For example, tricaine methanesulfonate has a 21-d withdrawal period before fish can be consumed as well as a costly. Therefore, there is a need for a reliable, a more cost-effective and safe anesthetic for use in fisheries research as well as aquaculture.

Recently, clove oil has received favorable reviews as an alternative fish anesthetic for a variety of fish species as well as for crustacean. Clove oil is used throughout the world for applications ranging from food flavoring to local anesthesia in the dentistry profession (Nagababu and Lakshmaiah, 1992). Clove oil is a natural substance from clove plant (*Eugenia caryophyllus*) containing 70-80% eugenol [2 -methoxy -4 -2 -(2 -propenyl) -phenol] (Ross & Ross, 1999) and its acceptable daily human intake levels have been established as 2.5 mg/kg (Anderson *et al*., 1997). Furthermore, eugenol is considered noncarcinogenic, non-mutagenic and a GRAS (General Recognized as Safe) substance by the FDA (Nagababu & Laksmaiah, 1992).

A number of studies have tested clove oil as an anesthetic in several aquatic animals both marine and freshwater species such as: rabbitfish *Siganus lineatus* Curvier & Valenciennes (Soto & Burhanuddin, 1995), channel catfish *Ictalurus punctatus* Rafinesque (Waterstrat, 1999), rainbow trout *Oncorhynchus mykiss* Walbaum (Keene *et al*., 1998), mullet *Valamugil cunnesis* (Durville & Collet, 2001), gilthead sea bream, *Sparus aurata* (Tort *et al*., 2002) and longfinned eel, *Anguilla reinhardtii* Steindachner (Walsh & Pease, 2002). In addition to, Erdmann (1999) suggested that clove oil can act as an alternative to cyanide use in the live reef fish industry. Clove oil is also considered less expensive compare to the other fish anesthetics such as tricaine methane-sulfonate and it can be purchased effortlessly throughout Southeast Asia countries. However, there are no study of clove oil for sea bass (*Lates calcarifer* Bloch) either its toxicity or as an aid in live fish transportation.

The Asian sea bass (*L. calcarifer* Bloch) is an important species for aquaculture in Southeast Asia. It is highly priced food fish in Asia. The culture of sea bass has been developed rapidly throughout the seventies and eighties. It was due to the successful technical development of hatchery-produced sea bass fry in Thailand, Australia as well as Indonesia.

The purposes of this study were to examine the safety and efficacy of clove oil as an anesthetic for fry of sea bass (*L. calcarifer* Bloch) and to determine whether clove oil is a suitable anesthetic for fry sea bass transportation.

Materials and Methods

Fish and anesthetic

The study was done at Department of Aquaculture, Faculty of Fisheries, Kasetsart University in March-July 2003. Asian sea bass (*L. calcarifer* Bloch.) fry (3.1±0.15 cm in length and 0.45±0.74 g in weight) were obtained from a hatchery. Before experiment, fishes were adapted in fiber glass tank filled sea water (15 ppt) for 2-3 weeks. Fish were fed live adult artemia. The fish were starved for 24 hrs prior to experiment.

Clove oil was purchased from Indonesia. Clove oil used in this experiment is a local

product. The active ingredient of clove oil is eugenol (95%). The density of clove oil is approximately 1 g/ml. Fresh solutions (based on the active ingredient) were prepared prior to each experiment conducted and were protected from sun and heat to limit photo and thermal degradation. In all experiments, clove oil was added directly to the water and the water was vigorously aerated for 5 min prior to the addition of experimental fish.

Experiment 1: The efficacy of clove oil as an anesthetic in sea bass fry

This experiment was divided into two studies i.e. the 24-h LC_{50} test and the efficacy test of clove oil on sea bass fry. The acute toxicity of clove oil to sea bass fry was determined following the methodology for static test (Parrish, 1985; APHA, 1998). Static bioassays were conducted in 45 l glass aquaria containing 40 l of sea water (15 ppt) and 20 test fish. The concentrations of clove oil used were chosen to give 0 and 100% mortality of test animals within 24 h based on the results of preliminary studies. There were triplicate aquaria at each concentration and for controls. Total mortality was measured for the 24 h-experiment. The 24-h LC_{50} estimate was calculated with the method as described by Litchfield & Wilcoxon (1949).

As the 24 hr LC_{50} test, the efficacy test of clove oil was conducted in static glass aquarium containing 40 l seawater (15 ppt) with continuous aeration. Fish were individually exposed to concentrations of 0, 5, 10, 15, 20 or 25 ppm clove oil for a period of 15 min, monitored for behavioral responses, then removed from anesthetic solution and placed in free anesthetic water to monitor recovery. Fifteen fish were used for each concentration. Following recovery, fish were returned to glass aquaria and monitored for either mortality or abnormality behavior for 7 days. The efficacy criteria were ability to handle fish within 3 min, fish recovery within 10 min, and survival of a 15 min exposure trial (Gilderhus & Marking, 1987). Stages of anesthesia were visually monitored, timed and classified (Table 1). Mean induction (time from stage 1 to 4) and recovery times were compared among concentrations of clove oil using one-way Analysis of Varians, followed by Tukey's Honestly Significant Difference multiple comparison procedure (Zar, 1984).

Experiment 2: Potential application of clove oil as a fish anesthetic in the closed bag transport of Asian sea bass fry

The experimental groups were divided into three groups. The first was a group contains treated fish with sedation concentration of clove oil (5 ppm). The second group was treated with anesthetized concentration of clove oil (20 ppm). Those concentrations were based on the result from previous experiment. The last group was control fish with no anesthetic treatment. Each treatment was carried out in triplicates. There were two studies of simulated transport based on duration of transport time and density of fish. First study was conducted in 4 h simulated transport with 50 fish per 1,000 ml sea water (15 ppt). The second was carried out in 8 h simulated transport with 50 fish per 500 ml sea water (15 ppt).

Table 1. Stage of anesthesia used for sea bass clove oil efficacy test

Stage	Characteristic behavior
Stage 1	Sedation: partial or total loss of reaction in response to external stimuli and equilibrium is normal.
Stage 2	Swimming ability disrupted and loss of equilibrium in which locomotion ceases but fish respond to pressure on the caudal peduncle.
Stage 3	Anesthesia: loss of reflex activity or failure to respond to strong external stimuli.
Stage 4	Medullary collapse: respiratory movements or opercular activity cease and fish death.
Recovery	Ability to remain upright, regain control of equilibrium and normal swimming behavior

The packing system involved polyethylene bags, each measuring 50x25 cm in size. Double polyethylene bags, one slipped into another, were used to ensure against water loss from perforations or leakage. Each bag was added 1,000 ml or 500 mi sea water (15 ppt) followed by clove oil and 50 fish. Clove oil was vigorously stirred into the packaging water before the fish were put in. The bags were then flattened to the water surface to expel the air, inflated with oxygen gas, sealed with rubber bands and finally, put it in the Styrofoam box. The Styrofoam box was left in the laboratory for the test period i.e. 4 and 8 hr.

After the end of simulated-transport, the bags were opened, water samples were taken and fish were transferred into the fiber glass tanks. Any dead fish were separated and counted with subsequent calculations of mortality levels. The survive fish were reared and observed for mortality and health condition for 7 days. Water samples were taken before and after transport for the measurement of total ammonia by Koroleff's Indophenol Blue method (APHA, 1998), nitrite and alkalinity by colorimetric method (APHA, 1998). The other water quality parameters were temperature, salinity (hand refracto-meter), dissolved oxygen (YSI Oxygen-meter), and pH (pH meter). The experiment used completely randomized design in triplicates. Mortality and the physicochemical parameters of the water were analyzed using analysis of variance and the Tukey test (Zar, 1984).

Results and Discussion

The efficacy of clove oil as an anesthetic in sea bass fry

The 24 hr LC_{50} and 95% confidence intervals of clove oil on sea bass fry was 30 (29.01 to 31.02) ppm with slope function of 1.079 (1.05 to 1.107). In the efficacy test, sea bass fry exposed to clove oil passed sequentially through the anesthetic stages as described above. Control fish swam normally without any abnormality and responded to stimuli by rapidly changing direction and avoiding the source of the stimulus. Changes in the swimming behavior (disorientation and erratic swimming), in gill ventilation, and in the color of fish (darkened skin) were observed within several minutes after addition of clove oil in the aquarium.

Table 2 shows the effects of clove oil at different concentrations on times required to induce each anesthetic stage and for recovery stage. Increasing the dosage of clove oil decreased the time required to induce each anesthetic stage. Recovery time also increased with clove oil concentration.

Fish exposed to 5 and 10 ppm of clove oil did not enter stage 2 or 3 during the 15-minutes exposure period. At those concentrations, fish were able to maintain their upright position in the water. However, fish exhibited less response to external stimuli such as pressure on the caudal peduncle or tapping in the glass aquarium compare to control fish. Some of tested fish exhibited erratic swimming at the first time exposed to clove oil.

Table 2. Induction and recovery times for sea bass fry exposed to various concentrations of clove oil for a period of 15 minutes

Concentration of clove oil (ppm)	Induction time (minute)			Recovery time (minute)
	Stage 1	Stage 2	Stage 3	
5	$10.06^{a} \pm 0.21$	$15.00^{a*} \pm 0.00$	$15.00^{a*} \pm 0.00$	$3.07^{a} \pm 0.04$
10	$7.93^{b} \pm 0.04$	$15.00^{a*} \pm 0.00$	$15.00^{a*} \pm 0.00$	$4.22^{b} \pm 0.05$
15	$5.02^{c} \pm 0.09$	$6.28^{b} \pm 0.07$	$7.64^{b} \pm 0.06$	$7.41^{c} \pm 0.07$
20	$2.04^{d} \pm 0.05$	$2.62^{c} \pm 0.23$	$3.15^{c} \pm 0.04$	$8.64^{d} \pm 0.05$
25	$1.88^{d} \pm 0.02$	$2.46^{d} \pm 0.08$	$2.90^{d} \pm 0.02$	$9.88^{e} \pm 0.04$

Note: In each column, means followed by different letters are significant different (α=0.05); Average weight of fish=0.45 ± 0.74 g and average length of fish=3.1±0.15 cm; * = No stage 2 or stage 3 was noted during the 15 minutes exposure time.

They moved up and down in the column of water and some of them remained in the water surface with less swimming movement during exposure period.

Mean times to stage 2 decreased significantly from 6.28 min at 15 ppm to 2.46 min at 25 ppm. Mean times required to induce stage 3 also decreased significantly from 7.64 min at 15 ppm to 2.90 min at 25 ppm. Nevertheless, all fish did not reach to stage 4 or *medullary collapse* during 15 minutes exposure period. At those concentrations, fish showed loss of equilibrium and lay flat on their sides on the bottom of aquarium.

Recovery times were concentration dependent. Increases in the concentration of clove oil produced increases in the time required for recovery. Mean recovery times for sea bass fry exposed to 15, 20 and 25 ppm of clove oil were 7.41, 8.64 and 9.88 minutes, respectively. No mortalities were recorded during the 15 minutes exposure time at various concentrations of clove oil as well as during 7 days post recovery.

Potential application of clove oil as a fish anesthetic in the closed bag transport of Asian sea bass fry

All fish survived throughout 4 hr of simulated transport at packing density of 50 fish per 1,000 ml sea water in all treatments as well as control. No mortality was also observed up to 7 days post transport in the 40-l aquaria. Table 3 shows the result of water quality parameters measuring at the initial and after 4 hr simulated transport.

The pH levels decreased in all treatments and control although no significant difference was noted among them. Free carbon dioxide in water increased significantly from 0 up to 25.97 ppm after 4 hr simulated transport. Maximum carbon dioxide level (25.97 ppm) was found at fish transported without clove oil. The dissolved oxygen levels in the water decreased significantly after 4 h simulated transport with minimum levels of 1.67 ppm were observed in the control group. In terms of total ammonia, an increase in concentration was observed in all cases before and after 4 hr simulated transport. Maximum total ammonia (2.08±0.01 ppm) was observed at the control treatment. Total ammonia levels in 5 and 20 ppm of clove oil treatment were slightly decreased compare to those in the control treatment.

In the second study of transport, all fish survived in the groups with 5 ppm clove oil after 8 hr simulated transport. However, survival rate was decreased in the control and 20 ppm clove oil treatment. The lowest survival rate (75.33%) was noted in the control treatment. However, fish transported with 20 ppm clove oil resulted in 94.67% of survival rate. There was no mortality during the 7 days post simulated transported.

Table 3. Water qualities in polyethylene bags containing 1,000 ml sea water (15 ppt), 50 sea bass fry and different concentrations of clove oil at initial and after 4 hr simulated transported at 25^0C

Water quality parameters	Initial measurement	After 4 hr simulated transport		
		Control (0 ppm)	5 ppm	20 ppm
pH	8.21 ± 0.08^a	6.66 ± 0.02^b	6.81 ± 0.72^b	6.76 ± 0.02^b
Free CO_2 (ppm)	0 ± 0.00^a	25.97 ± 0.57^b	22.64 ± 0.88^c	22.30 ± 0.33^c
Dissolved oxygen (ppm)	14.9 ± 0.08^a	1.67 ± 0.18^b	4.53 ± 0.09^c	6.40 ± 0.10^d
Total alkalinity (mg/l $CaCO_3$)	71 ± 0.57^a	74 ± 0.57^a	72.3 ± 0.88^a	73 ± 0.57^a
Total ammonia (ppm)	1.45 ± 0.02^a	2.08 ± 0.01^b	1.99 ± 0.04^c	1.92 ± 0.01^d
Nitrite (ppm)	0.002 ± 0.00^{ab}	0.002 ± 0.00^{ab}	0.0018 ± 0.00^a	0.0023 ± 0.00^b

Note: Results are mean of three replicates with standard error; In each row, different superscript letters represent significantly different mean values (α = 0.05).

Table 4. Water qualities in polyethylene bags containing 500 ml sea water (15 ppt), 50 sea bass fry and different concentrations of clove oil at initial and after 8 hr simulated transported at 25^0C

Water quality parameters	Initial measurement	After 8 hr simulated transport		
		Control (0 ppm)	5 ppm	20 ppm
pH	9.10 ± 0.01[a]	6.33 ± 0.01[b]	6.46 ± 0.01[c]	6.57 ± 0.04[d]
Free CO_2 (ppm)	0 ± 0.00[a]	28.96 ± 0.00[b]	32.63 ± 0.8[b]	32.62 ± 0.8[b]
Dissolved oxygen (ppm)	14.8 ± 0.25[a]	2.27 ± 0.46[b]	5.77 ± 0.12[c]	5.43 ± 0.06[c]
Total alkalinity (mg/l $CaCO_3$)	74.3 ± 0.33[a]	75 ± 0.57[a]	72.6 ± 0.45[a]	74 ± 0.57[a]
Total ammonia (ppm)	1.34 ± 0.03[a]	2.65 ± 0.03[b]	1.86 ± 0.01[c]	1.77 ± 0.01[c]
Nitrite (ppm)	0.019 ± 0.00[a]	0.053 ± 0.00[b]	0.02 ± 0.00[ac]	0.02 ± 0.00[ac]

Note: Results are mean of three replicates with standard error; In each row, different superscript letters represent significantly different mean values (α = 0.05).

Table 4 shows the initial measurement of water quality parameters and after 8 hr simulated transport. Decreasing pH levels differed significantly among the control and treatment groups. After 8 hr simulated transports, free carbon dioxide reached 32.62 ppm as the maximum level in either 5 or 20 ppm clove oil treatment in which those values were slightly higher than those in control group. However, the minimum dissolved oxygen (2.27±0.46 ppm) was found in the control group. As in 4 hr transport, total ammonia increased significantly after 8 hr simulated transport. Maximum level of total ammonia (2.65±0.029 ppm) was noted in the control group and there was a significantly difference between treated and control group.

In term of feeding behavior, either in 4 or 8 hr transport, control fish consumed artemia immediately after reared back to the aquaria. Nevertheless, treated fish (5 and 20 ppm) required within 10 to 30 minutes to consume artemia offered. There was no abnormality both in feeding activity and behavior among experimental groups during 7 days post transport.

In present study, clove oil appears to act as an anesthetic in sea bass fry. Sea bass fry exposed to clove oil progressed sequentially through the stages of anesthesia. The estimated 24 hr LC_{50} value of clove oil on sea bass fry (0.5 g in weight and 2.7 cm in length) was 30 ppm. Keene *et al.* (1998) reported that the 0.5-96 hr LC_{50} of clove oil for rainbow trout (20 g in weight and 12 cm in fork length) was 65-9 ppm. Meanwhile, Taylor & Roberts (1999) noted that the 10 min LC_{50} of clove oil for juvenile white sturgeon, chinook and coho salmon were 526, 62 and 96 ppm, respectively. The differences between those values indicate a tolerance difference of clove oil among species tested.

There is no simple definition of efficacy of anesthetics in fish, and many papers that were published regard efficacy as the ability to handle the fish (Gilderhus & Marking, 1987). At concentrations of 10 ppm or less, stage 3 anesthesia, which fish lost their equilibrium and reflex, was not observed in any sea bass fry after 3-min exposure time, nor did they reach stage 3 within the 15-min exposure period. In this experiment, clove oil was an effective anesthetic for sea bass fry at concentration ≥ 15 ppm. The fish entered behavioral stage 3 within 7.6-2.9 min for concentrations of 15-25 ppm. It appears that clove oil has shown to immobilize fish effectively at low dosage. This also concurs with several studies (Keene *et al*., 1998; Stehly & Gingerich, 1999; Cho & Heath, 2000, Durville & Collet, 2001).

The results from induction time indicated that clove oil, at 20 and 25 ppm, meet both Marking & Meyer's first criterion (1985) and Gilderhus & Marking's criteria (1987). Meanwhile, regarding recovery time, sea bass fry exposed to 15-25 ppm of clove oil demonstrated recovery within 7-9 min. It indicates that anesthetizetion with clove oil required longer recovery

time. It met the criteria from Gilderhus & Meyer (1987) in term of recovery time.

In present study, clove oil can act as an aid in sea bass fry transport. At 5 ppm clove oil treatment, fish revealed slow swimming activity without loss of equilibrium or reflex. Whereas at 20 ppm, fish exhibited loss of reflex and lay flat on the bag bottom throughout the 4 hr transport. In 8 hr transport, however, clove oil appears to be effective only in the half of experiment. This was probably caused by the volatile characteristic of clove oil or the high fish density in the bag.

In 4 hr transport, both 5 and 20 ppm clove oil reduced fish activity judging from the reduction of oxygen consumption, ammonia and carbon dioxide levels but no improvement on survival rate. However, in 8 hr transport and high packing density, survival rates were improved by application of both 5 and 20 ppm clove oil. Based on the result, an appropriate concentration of clove oil for sea bass fry transportation should be 5 ppm. This concentration caused no mortality, ensured fish stay calm without loss of equilibrium and reduced the metabolic rate causing reduction of oxygen consumption, carbon dioxide, and total ammonia in the water.

In conclusion, clove oil does appear to have promise as an effective and safe anesthetic for use on food fishes, e.g. sea bass, carp and grouper. It is a natural substance, inexpensive and easy to obtain particularly in developing country. In addition, application of clove in fish transport can reduce metabolism rate resulting in reduction of oxygen consumption, ammonia and carbon dioxide accumulation and improvement of survival rate.

Acknowledgements

The study has been carried out with financial support from a grant-in-aid from Loan Asian Development Bank (ADB) No 1570/1571 (SF)–INO. We want to thank Aquaculture Department, Faculty of Fisheries, Kasetsart University for all facility.

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