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Comparison of the Effects of Clove Oil as an Anesthetic on Blood Biochemical Profiles of *Rutilus frisii Kutum* and *Rutilus rutilus caspicus*

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Abstract: The efficacy of clove oil as an anesthetic was compared in *Rutilus frissi kutum* and *Rutilus rutilus caspicus*. The time loss of equilibrium, induction time, blood biochemical profiles, of *Rutilus frisii Kutum* and *Rutilus rutilus caspicus* anaesthetized with clove oil (10 and 20 mg l^{-1}) were evaluate. Fish were divided into two groups, one sampled 10 min after anesthesia and a second, sampled 24 h after anesthesia. The obtained results suggested that the induction times decreased with increasing doses in both spices. Also, Time loss of equilibrium (A3) in 10 and 20 mg/l was significantly lower in *Rutilus frisii Kutum* and the induction time (A5) in 10 mg/l did not show any significantly differences and in 20 mg/l was greater in *Rutilus rutilus caspicus*. A significant increase ($P \leq 0.05$) in blood plasma glucose immediately after the 10min clove oil anesthesia was observed. Other parameters were not altered. On the basis of this experiment, it appears that the use of clove oil attenuated the stress response and the use of clove oil at a concentration of 10 and 20 mg/l does not cause irreversible damage in *Rutilus frisii Kutum* and *Rutilus rutilus caspicus*.

Key words: *Rutilus frisii Kutum* · *Rutilus rutilus caspicus* · Clove Oil · Anesthesia

INTRODUCTION

The use of anti-stress agents is a common practice in modern aquaculture. Such substances are used to induce anesthesia during handling, sorting, tagging, artificial reproduction procedures and surgery, thus reducing stress-induced problems such as reductions in feeding and immune function [1]. Stress-related changes in blood chemistry occur within a few seconds when fish are disturbed [2]. These include changes in plasma hormones, energy metabolism and electrolyte balance. Fish culturists and fish biologists make use of blood chemistry indices for evaluation of fish stress responses, nutritional condition, reproductive state, tissue damage due to handling procedures and health status [3].

When choosing an anesthetic, a number of considerations are important, such as efficacy, cost, availability and ease of use, as well as toxicity to fish, humans and the environment [4] and the choice may also depend on the nature of the experiment and species of fish [5]. So far, a number of different anesthetics have been used or evaluated for aquaculture applications.

Clove oil is obtained by distillation of the flowers, stems and leaves of the clove tree (*Eugenia aromatic* or *caryophyllata*). In addition to its worldwide use as a food flavoring, it has also been employed for centuries as a topical analgesic in dentistry [6]. It is considered superior to a number of anesthetics, such as quinaldine, benzocaine and MS-222 [7], the latter being the only approved anesthetic for fish in the US. Furthermore, the longer recovery time exhibited by fish anesthetized with clove oil [8] may be an additional advantage in activities such as morphological evaluations, acquisition of tissue biopsies and strip-spawning, where long handling periods outside the water are involved [9].

Clove oil, its major constituent eugenol and the synthetic iso-eugenol appear to fulfill adequately the above criteria and a great number of studies have been undertaken in the last decade in order to evaluate its efficacy in a variety of species and applications. Its active ingredient, eugenol (4-allyl-2-methoxyphenol), has been reported to comprise between 70% and 90% by weight of clove oil [10].

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Eugenol is rapidly absorbed and metabolized after oral administration and it is almost completely excreted in the urine within 24 h with no apparent ill effects [11]. Thus, eugenolhas long been considered safe for laboratory use [12].

Building on the initial studies of and Hikasa *et al.* [13], a suite of research has been conducted that characterizes the dose responses to clove oil for a variety of cultured fishes (e.g., Asian sea bass *Lates calcarifer* [14]; rainbow trout, *Oncorhynchus mykiss* [8, 10]; Atlantic salmon, *Salmo salar* [15]. Collectively, the range of studies available suggests that clove oil is an effective alternative for the sedation of fish and may in fact have several benefits over other methods including its low cost. Interestingly, of those studies conducted, most have assessed high clove oil concentrations that result in deep sedation, loss of equilibrium and loss of reflex reactivity.

The investigation of any impact that eugenol may have on several frequently used blood chemistry indicators is necessary in order to evaluate its potential use for routine aquaculture and fisheries research.

The primary aim of this study was to compare the effects of clove oil using 2 concentrations of clove oil on the time of anesthesia and plasma dynamics in *Rutilus frisii Kutum* and *Rutilus rutilus.*

Caspian kutum, *Rutilus frisii Kutum* and *Rutilus rutilus*, are endemic fish of Caspian Sea and its populations generally recorded along near the coast, from the Trek River the north to the southern part.

MATERIALS AND METHODS

Fish: The experiments were undertaken between September and November 2010, using produced at the Institute of aquaculture University Agriculture Science and Natural Resources, Gorgan, Iran. *Rutilus frisii Kutum* and *Rutilus rutilus* (3±0.38 and 3±0.7 g) were used in the study. Prior to the study, fish were maintained ingroups in 450-L tanks in an indoor facility for 3 month. Environmental conditions were monitored and maintained within a narrow range of values. A 12-hour-light: 12-hour-dark cycle was maintained. Fish were fed a pelleted diet formulated for once daily. All fish were healthy prior to and throughout the study. Fish fasted and without human contact or disturbance for 24 h before the start of the study. The average water temperature and oxygen level during the experimental period were 23±0.2°C and 12.1±0.8 mg/l, respectively. Temperature and dissolved oxygen were monitored daily and water renewal was 25% per a day.

Anesthesia: Clove oil is poorly soluble in cold water and therefore was initially dissolved in 94% ethanol (ratio of clove oil:ethanol, 1:9). To examine the effect of ethanol exposure, a group of 10 fish of each species was transferred by net into a 1-l bucket with 3.38 mg/L of ethanol and were observed for 15 min. The end points monitored in the anesthesia experiments were modified from earlier studies [5, 10] and were (a) complete loss of equilibrium (stage A3) and (b) complete loss of responsiveness to tactile stimuli (stage A5, Table 1).

Experimental Procedure: We had three groups in each species:

- Control - no anesthetic, blood sampled prior to the treatment of anaesthetized groups.
- Three groups of two fish species with blood sampled immediately after anesthesia and designated as: clove oil (10 and 20 mg/l)
- Three groups with blood sampled after 24 h anesthesia and designated as: clove oil (10 and 20 mg/l)

There were no mortalities in the study. Fish of each species separately (n =10) were taken from the acclimation tanks and were transferred (two at a time) into the anesthetic aquarium and the time to reach stages A3 and A5 was recorded by two observers.

Table 1: Stages of Anesthesia From Anesthesia Employed As Endpoints In The Present Study Modified From Summerfelt and Smith [5] and Keene *et al.* [10]

Stage	Description	Notable behavior
Anesthesia		Total loss of equilibrium,
A3	Loss of equilibrium	pectoral fins moving,
A5	Deep anesthesia	regular opercular ventilation
		No movement, loss of responsiveness to tactile stimuli, slow and irregular opercular ventilation

The behavioral response to anesthesia appeared to be the same between the two species, both species continued to swim after losing equilibrium.

Biochemical Blood Profiles: Blood was taken into heparinized capillary tubes by cutting caudal vein. Hematocrit was calculated after centrifugation of microhaematocrit tubes (4,000 g for 5 min, Sigma 1-15 microcentrifuge). Because blood of fish wasn't sufficient, blood of 25 fish was collected (0.5-1.5 ml) in Eppendorff vials and centrifuged (9.000 g for 13 min, Sigma 1-15 microcentrifuge). Then plasma was collected and stored at -80°C for future analysis. Glucose, chloride and calcium plasma concentration in plasma were measured by Olympus 600 Medicon Analyzer, using Heokinase enzymatic UV and Arsanazo III photometric color tests, respectively. Plasma Na^+ and K^+ was determined using Hitachi 911 instrument.

Statistical Analyses: Statistical analysis of data was performed by mixed analysis of repeated measurement in SAS software. The significance level was chosen as $P \leq 0.05$ in all tests.

RESULTS

An ideal anesthetic for fish should induce anesthesia in less than 3 to 5 min, with total loss of equilibrium and muscle tone, should allow an uneventful and rapid (i.e. less than 10 min) recovery, should leave low tissue residues and be safe to usersas well as be inexpensive and easy to use [16].

Exposure of fishes to ethanol (i.e., the anesthetic solvent) did not induce anesthesia, or any apparent modifications in the behavior of the fish. Opercular ventilation rate also was not affected (data not shown), suggesting that the concentration of ethanol used had no, or negligible effect on the fish within the exposure time used in the study. Finally, no mortality was observed during the 5-day period following exposure and the fish were feeding well within 1 day after treatment. Fries continued to swim after losing equilibrium and for recovery, they first regained equilibrium and then begun swimming slowly.

All fish used in the present study were healthy as was indicated by their activity and exterior appearance. No mortality was observed during the acclimatization period.

At concentration of 10 and 20 mg/l all fish reached stage A5 anesthesia within 3 min exposure (Table 2). Induction of anesthesia was uneventful in all fish. Induction time decreased with increasing concentrations, being significantly concentration-dependent ($P \leq 0.05$) in both species. Time loss of equilibrium (A3) in 10 and 20 mg/l was significantly lower in *Rutilus frisii Kutum* and the induction time (A5) in 10 mg/l wasn't any significantly difference and in 20 mg/l was greater in *Rutilus rutilus caspicus* (Table 2).

The biochemical profiles of *Rutilus frisii Kutum* and *Rutilus rutilus caspicus* are given in Table 3. It shows the

Table 2: Efficiency of 10 And 20 Mg/L Clove Oil On Least Square Mean Of Total Loss of Equilibrium And Deep Anesthesia In *Rutilus frisii Kutum* and *Rutilus rutilus caspicus*

species	Concentration(mg/l)	A3(min)	A5(min)
Rutilus rutilus caspicus	10	1.3[a]	1.93[a]
	20	0.53[c]	1.32[b]
Rutilus frisii Kutum	10	0.93[b]	2.9[a]
	20	0.36[d]	1.03[c]

Data at the same column within the same superscript letters are not significantly different between different anesthetic treatments ($P \leq 0.05$)

Table 3: Comparative of Least Square Mean Of Biochemical Blood Profile Between Rutilus *frisii kutum and Rutilus rutilus caspicus*

	0			20mg/l					10mg/l					
	Control			10min		24h			10min			24h		
	Rutilus rutilus		*Rutilusfrisii*	*Rutilus rutilus*		*Rutilusfrisii*	*Rutilus rutilus*		*Rutilusfrisii*	*Rutilus rutilus*		*Rutilusfrisii*	*Rutilus rutilus*		*Rutilusfrisii*
Hem(%)	40.6	37.1	42	36.8	42	37.3	41.6	36.3	40.6	36	0.06	0.001*	0.005*	0.000*	0.005*
Glu(mg/dl)	47.3	58	121	88.5	75.3	57.6	123	96.8	75	58.3	0.000*	0.000*	0.000*	0.000*	0.000*
Ca^{++}(mg/dl)	9.1	8.5	9.8	8.3	9.2	8.1	9.4	8.6	9.2	8.5	0.157	0.000*	0.000*	0.02*	0.04*
Na^{++}(mmol/l)	140	136.3	147	139	141.6	134.6	147.6	140.6	142	135.6	0.02*	0.000*	0.000*	0.000*	0.000*
K^+(mmol/l)	5.4	6.6	6.4	7.4	5.7	6.3	6.5	7.5	6	6.4	0.000*	0.03*	0.5	0.03*	0.8
Cl^-(mmol/l)	135	121.3	134.3	126.6	132.6	127.6	134.6	127.6	132.3	127.5	0.000*	0.000*	0.001*	0.000*	0.002*

* Significance levels observed Are $P \leq 0.05$ incomparison between 2 species

Table 4: Effect of Species on Different Concentration

		0	20 mg/l		10mg/l	
Indices	Species	Control	10min	24h	10min	24h
Hem	*Rutilusfrisii*	37.1[a]	36.8[a]	37.3[a]	36.3[a]	36[a]
	Rutilus rutilus	40.6[a]	42[a]	42[a]	41[a]	40.6[a]
Glu	*Rutilusfrisii*	58[c]	88.5[b]	57.6[c]	96.8[a]	58.3[c]
	Rutilus rutilus	74.3[b]	121[a]	75.3[b]	123[a]	75[b]
Ca^{++}	*Rutilusfrisii*	8.5[a]	8.3[a]	8.1[a]	8.6[a]	8.5[a]
	Rutilus rutilus	9.1[a]	9.8[a]	9.2[a]	9.4[a]	9.2[a]
Na^{++}	*Rutilusfrisii*	136.3[a]	139[a]	134.6[b]	140.6[a]	135.6[a]
	Rutilus rutilus	140[b]	147[a]	141.6[b]	147.6[a]	143[b]
K^{+}	*Rutilusfrisii*	6.6[a]	7.4[a]	6.3[b]	7.5[a]	6.4[a]

Data at the same column within the same superscript letters are not significantly different between different anesthetic treatments ($P \leq 0.05$)

comparative of least square mean of blood profile in two species at different concentrations and times. Blood profile of two species in both concentrations and control group (10 and 20 mg/l) were significantly different ($P \leq 0.05$) in different times.

Effect of species on different concentration is shown on Table 4. The level of glucose was significantly ($P \leq 0.05$) greater with anesthesia after 10 min and decrease 24h after it compared with controls. Immediately after clove oil (10 min) anesthesia, *Rutilus rutilus* showed significantly higher ($P \leq 0.05$) concentration of sodium compared with the control group and with all other treatments after 10 min and return to its normal level 24 hour after anesthesia. The exposure to the anesthetic at a concentration of 10 and 20 mg/l had no effect on the haematological indices studied (Hem, Ca, Na, Cl, K).

DISCUSSION

Recent work has demonstrated clove oil (eugenol) to be a suitable anesthetic for aquacultural and fisheries use [10], although it is important to note that it has not been approved for such applications in most countries at the present time [17]. This is due primarily to the lack of animal and human safety testing necessary to support applications for regulatory approve. As a result, research remains the only safe way to evaluate the efficacy of an anesthetic in a particular species and establish the minimum concentration that produces desirable anesthetic effects on it.

A variety of factors, including species, size, body weight, gill surface area to body weight ratio, lipid content, sex, sexual maturity, physical condition, health state and stocking density, as well as temperature, pH, salinity and oxygen and mineral content of the water may affect the anesthetic process in fish [18-21]

In the present study clove oil at concentrations of 10 and 20 mg/L induced anesthesia rapidly in *Rutilus frisii Kutum* and *Rutilus rutilus caspicus.* In general the time loss of equilibrium was lower in *Rutilus frisii Kutum.* It is not possible to explain the different responses in induction time and the time loss of equilibrium. These differences documented in the present study underline the need to examine the response of each fish species of interest to anesthetic exposure, prior to its recommendation for large-scale use. In the present study, induction time decreased and with increasing concentrations. This is in agreement with other reports on the efficacy of clove oil in various species [19, 20,22].

As both 10 and 20 mg/L of clove oil induced safe and effective anesthesia, we suggest that the drug should be used in both species at a concentration of 10 mg/L for all but the most painful procedures where a concentration of 20 mg/L should be preferred. However, even the lowest concentration should not be considered absolutely safe when used to fish of a compromised health status, as it is well known that anaesthetizing animals that have infections usually results in increased mortality and morbidity [21].According to our results, lower concentrations of clove oil, e.g. 10 or 20 mg/L, could be used as sedatives in *Rutilus frisii Kutum* and *Rutilus rutilus caspicus*.

The analysis of the blood parameters is one of the most valuable methods of modern diagnostics [23] and can provide important information about the internal environment of the organism [23-25]. In our experiments, a significant increase ($P = 0.05$) in blood plasma glucose immediately after the 10-min clove oil anesthesia was observed. Rapid increases in plasma glucose are mediated by the release of catecholamines, which increase (presumably in response to the hypoxia caused by cessation of respiration) in the plasma of anaesthetized

fish [26, 27]. Our findings agree with those of Velisek *et al*. [28] who observed an increase in plasma glucose in perch (*Perca fluviatilis* L.) following anesthesia. Also Sladky *et al*. [29] observed increased in glucose concentration in red pacu (*Piaractus brachypomus*) flowing anesthesia with clove oil. Also clove oil increased glucose in another experiment with rainbow trout [3].

Increases in blood sodium (significantly in *Rutilus rutilus caspicus*) and potassium (no significant) concentrations were observed when fish were exposed to anesthetic. This could be attributed to retention of sodium or potassium by the gills to compensate for loss of hydrogen ions, muscle contractions causing movement of sodium or potassium ions out of myocytes into plasma, release of catecholamines into the circulation, which stimulated Na^+-H^+ exchange in RBCs to maintain constant pH [30], or increases caused by RBCs lysis during the collection of blood samples [29]. The modest electrolyte alterations in the fish also could be explained by normal ion diffusion in the gills, because a flux of sodium and potassium ions of this small magnitude could happen with many physiologic or environmental manipulations [31].

The heamatocrit measurement in *Rutilus frisii Kutum* and *Rutilus rutilus caspicus* didn't have any significant difference exposure to anesthesia. Farahi *et al*. [32] also reported no changed in haematocrit, after anesthesia with different concentrations of clove oil in *Rutilus frisii Kutum*.

However, the final choice of anesthetic must take into account legislation, availability, cost-effectiveness, ease of use and safety for the user and the environment. Overall, from the five physiological parameters we measured, five responses were prevented (haematocrit, sodium, chloride and potassium) and one were altered (Glucose). In conclusion, the results obtained from the present study demonstrate that clove oil can be used as an effective and efficient agent for anesthesia in the aquaculture of *Rutilus frisii Kutum* and *Rutilus rutilus caspicus*.

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