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The Anesthetic Effects of Clove Essence in Persian Sturgeon, *Acipenser persicus*

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Abstract: This work was carried out in order to examine the efficacy of clove essence as an anesthetic on Persian sturgeon, *Acipenser persicus*. Also an experiment was carried out to investigate if clove essence anesthesia suppressed the normal plasma cortisol and increased glucose level in this species or not. The Effects of clove essence on hematological parameters (Hb, Hct and WBC) was examined as well. Three different concentrations of clove essence (200, 300, 400 ppm) combined the two water temperature (20 and 25°C) were assessed. From one trial (concentration: 300ppt and temperature: 25°C), blood samples were taken on 0 (immediately upon placement in recovery tank), 1,6,24 and 72 hours post recovery in order to evaluate physiological responses. Results indicated that anesthetizing with different concentrations of clove essence (200, 300, 400 ppm) combined the water temperature (20 and 25°C) rapidly occurred in less than 1.5 minute after exposure to the clove essence. After 72h both, blood glucose and cortisol reach the level similar to the control group which was before applying clove essence. Hematological parameters after some fluctuation reached its level the same as control group of the experiments. It was concluded that anesthetic effects of clove essence dosages, the combination of 400 ppm and 24°C was the best treatment for anesthetizing as well as recovery.

Key words: Acipenser % Clove % Anaesthesia % Cortisol

INTRODUCTION

In fisheries and aquaculture, anesthetics are helpful for reducing the stress caused by handling [1], transportation, Artificial insemination [2], sampling [3,4], tagging [5] and surgical preparations for physiological investigations [6].

Choosing an anesthetic must be attributed to several characteristics including its efficacy, availability; cost-effectiveness; ease of use; nature of the study; and safety for the user including fish, humans and the environment [7-9].

Since fish breathe through gills rather than lungs, anesthetic agents are greatly inhaled with gills. As a result, anesthesia must be added to the tank water and delivered through an aquatic medium [10]. Therefore, the relationship between the epithelium surface of the gill and the body volume as well as thickness of epithelium affect the efficacy of anesthetics [5, 11]. The other biological factors, including species, the stage of life cycle and age, size and weight, lipid content, body condition and disease status also influence the metabolic rate and therefore the pharmacokinetics of the anesthetic compound [8].

Water condition, such as temperature and pH can also affect the efficacy of an anesthetic solution on species [12].

Clove oil, natural oil derived from the clove plant, *Eugenia caryophyllata*, has some of the characteristics considered for an ideal anesthetic agent. The active ingredient of clove oil is eugenol (4-allyl-2-methoxyphenol), a phenolic compound [8, 10], which inhibits the prostaglandin H synthase (PHS) and result in analgesic effects of clove oil [13] What's more, the eugenol-based anesthetics are effective at low dosages, inexpensive, easily obtained and capable to reduce stress. They are also organic substances safe for both environment and user [2, 8]. Therefore, it could be a promising anesthetic agent in aquaculture and many studies have done to evaluate its efficacy on some species [7-9, 13-30].

Clove essence in Iran is used as an effective agent for anesthetizing fish in aquaculture facilities in order to mitigate the handling stress due to the grading, transporting and artificial spawning [31].

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Persian sturgeon is an endangered species [32]. It is spawned artificially in aquaculture facilities with the aim of restocking to improve its population. So some studies were done on effects of anesthetic agents on brood stocks and fries [33]. Raising sturgeons for producing broodstocks in order to reduce dependency on natural populations is very promising [34]. During culturing and breeding practices, stressful functions such as handling and transportation might affect its survival and growth, so using anesthetic agents could be helpful [35].

In order to examine the efficacy of clove essence as an anesthetic on Persian sturgeon, *Acipenser persicus*, an experiment was conducted. Also, the effect of clove essence anesthesia on the normal plasma cortisol and glucose values and hematological parameters were investigated as well.

MATERIALS AND METHODS

Fish and Rearing Condition: Experiments were conducted on juvenile Persian sturgeon (average weight: 148.7433±22.62g) produced at the Institute of Aquaculture of the Marjani for Sturgeon, Golestan, Iran. Prior to the study, fish were maintained in groups in 150-L aquariums in an indoor facility; fish had been maintained in this facility for more than 2 years. For the purpose of the study, fish were housed separately in experimental aquaria and acclimated to it for a minimum of 2 weeks. The aquaria shared a common source of water with a steady temperature of 25°C. Throughout the acclimatization period and during the experiment; environmental conditions were monitored and maintained within a narrow range of variable. Fish were kept under natural photoperiodic conditions, fed on hand with handmade pellet and fasted for 24 h prior to each experiment.

Anesthesia Preparation and Experiment: First part of the experiments was anesthetic effects. Before beginning the study, a pilot experiment was conducted and found that the characteristics defined the induction of different stages of anesthesia and recovery (Table 1) is similar to those reported previously by Iwama *et al*. [40]. Three different concentrations of clove essence (200,300,400 ppm) were chosen according to the previous scientific papers [add references] and previously pilot study. For preparation the desired dose of clove essence, a 10% stock solution (1 ml clove essence + 9 ml of maintaining water) was made. To make a 200 ppm solution, 2 ml of the stock solution were mixed with one liter of water and this procedure applied for the other two concentrations [2, 36].

Since many aquaculturists and clinicians add anesthetic agents directly to water baths to achieve the desired dose [23], the prepared clove essence solution was applied into water.

As far as diseased or weakened animals are much more susceptible to anesthetic treatment, six healthy Fish were anesthetized by immersing them in a bath containing anesthetic agent so that it is absorbed through the gills and rapidly enters the blood stream. For examination the effects of temperature, the water temperature (20 and 25°C) and anesthesia concentrations were combined. The fishes were placed through knotless dip net in an aerated container containing the holding tank water [10]. Aeration provided extra oxygen required during induction which causes increased respiration. To prevent abrading the skin of the fish, the handler wore wet latex gloves and gently transferred the fish into the container. When an anesthetic is first administered (induction), fish may go through an excitement phase, as inhibitory neurons are depressed before achieving anesthesia and becoming hyperactive for a few seconds [23], so a glass cover on the induction tank was used to make the anesthetic stages visible for the operator.

For the anesthetic effect, a video cassette recorder (DSC-W80, Japan) was used to record fish behavior for subsequent analyses [16]. Two observers made decision using the Table 1, according to the Iwana *et al*. [40].

Immediately after fish in each trial reach stage S , on a wet towel were weighed; total length was recorded and placed individually within 1 min into a recovery aquarium.

Table 1: Stages of Anesthesia and Recovery

Stages of Anesthesia	Description
I	Loss of equilibrium
II	Loss of gross body movements but with continued opercular movemets
III	As in stageII with cessation of opercular movements
Stages of Recovery	**Description**
I	Body immobilized but opercular movements just starting
II	Regular opercular movements and gross body movements beginning
III	Equilibrium regained and preanesthetic appearance

From Iwana *et al*. [40], modified by Ackerman *et al*. [12]

The recovery tank used the same water as anesthetic bath (at a similar temperature and chemistry) supplied with flow-through water at a high exchange rate to ensure that fish were always in contact with clean water. Water quality was carefully controlled during the experiment.

Anesthetic and recovery times to stage A3 and R3, respectively, were recorded from the time placing the fish in Anesthetic and recovery tank to the nearest second using an electronic stop-watch [9].

Physiological Experiment: For the second part of the experiment, from one trial (concentration: 300ppt and temperature: 25°C), apart from anesthetic experiment, five individual (average weight±sd: 146.88±17.364g) netted and bled serially at the times: 0 (immediately upon placement in recovery tank), 1,6,24 and 72 hours post recovery. Before using anesthesia, a group of fish bled as a control (C). For physiological responses, Blood (4 ml) was collected within 2 min of the fish being captured from the caudal vasculature using a syringe [29]. The blood samples were divided into two aliquots, one part was transferred to a 2 ml vacationer tube containing heparin sodium, shook for 2 minutes gently and stored in refrigerator prior to hematological analysis. Oxygen transport characteristics (hematocrit, hemoglobin) as well as White Blood Cells (WBC) were analysed [4]. The other part of aliquots transferred into a 1.5 ml microcentrifuge tubes and centrifuged for 15 minutes at 3000g [37] at 4°C. The plasma removed and transferred to another 1.5 ml microtube and stored frozen at -70 °C until subsequent analysis for metabolite concentration, cortisol and glucose [4].

RESULTS

A summary of the average time to Anesthetic stages at each of the tested dosages associated with the water temperature is presented in Table 2. Response time at tested dosages along with water temperature was rapidly occurred in less than 1.5 minute after exposure to the clove essence. All experimental fish were successfully revived and no mortalities observed by 72 h post-treatment.

Mean plasma cortisol concentration was 17.88±1.3 mg/ml for the control group; this was before the beginning of the experiment. Upon transporting anesthetic fish into recovery tank, plasma cortisol concentration upgraded to 121.58±14.85 mg/ml. Although after 72h decreasing in mean plasma cortisol concentration was revealed, it did not reach the similar concentration to that of the control. However, mean plasma cortisol concentration were not significantly ($p<0.05$) different between 72h and control group (Figure 1).

Plasma glucose concentration was 46.6±4.16 mg/dl I n control group and elevated upon placement in recovery tank. Nevertheless, elevating plasma glucose concentration stopped and alleviating started until 72h which was no significantly different among control, 24h and 72hgroups (Figure 2).

Oxygen transport characteristics in bloods as well as WBC analyzing are demonstrated in Figure 3, 4 and 5.

Table 2: Effects of clove essence and water temperature on anaesthesia and recovery of persian sturgeon

	Anesthetic time (s)				Recovery time (s)			
Dose (mgLG[1])	20°C		24°C		20°C		24°C	
200	168±51.5		136±38.5		181±60.2		144±66.86	
300	158±29.5		122±19.5		217±96.4		140±9.5	
400	121±28.9		110±30.2		223±33.79		159±23.21	
Two-way Anova								
	DF	Mean square	*F*-value	*p*-value	DF	Mean square	*F*-value	*P*-value
Dose	2	2784.92	2.35	0.12	2	1645.24	0.52	0.61
Temperature	1	4118.64	3.47	0.79	1	21312.96	0.67	0.01
Interaction	2	354.79	0.29	0.74	2	835.84	0.26	0.77

[1]Each value is mean±standard deviation (n=6).Values are not significantly different (P?0.05)

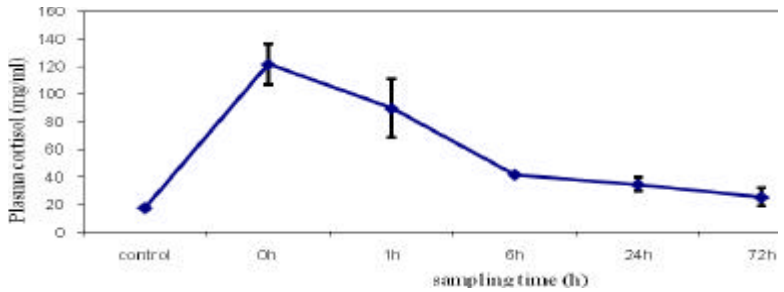

Fig. 1: Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma cortisol on the bars indicate statistical significance between treatment and control groups at a sampling time

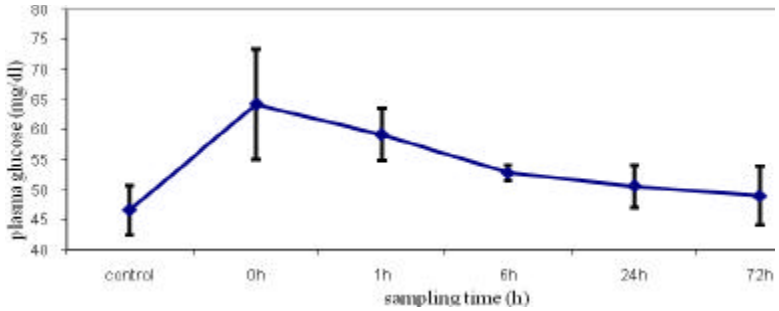

Fig. 2: Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma glucose on the bars indicate statistical significance between treatment and control groups at a sampling time

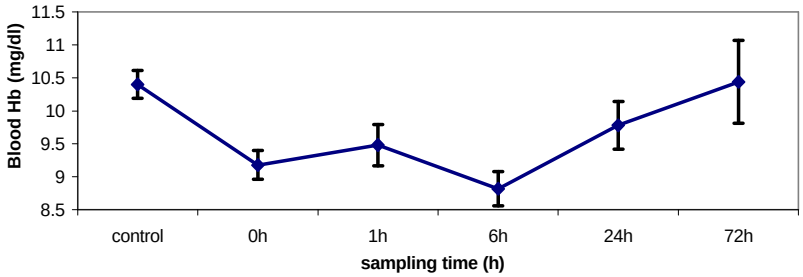

Fig. 3: Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma hemoglobin on the bars indicate statistical significance between treatment and control groups at a sampling time

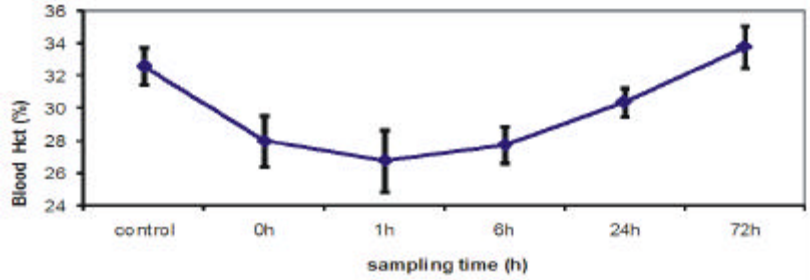

Fig. 4: Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma haematocrite on the bars indicate statistical significance between treatment and control groups at a sampling time

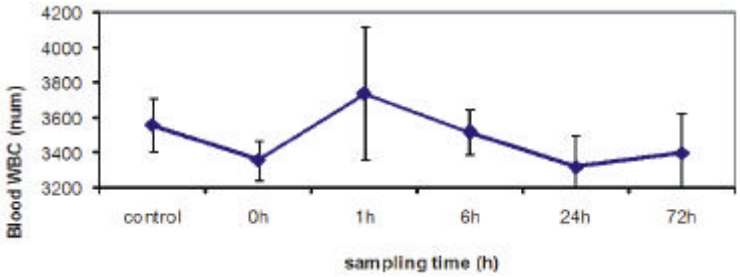

Fig. 5: Post-recovery Physiological measurement (mean±SD) of Persian sturgeon, *Acipenser persicus*, Different letters of plasma white blood cells on the bars indicate statistical significance between treatment and control groups at a sampling time

DISCUSSION

It was shown that the Persian sturgeon exposed to the clove essence tested concentrations in the present experiment; get in anesthetic phase less than 168 seconds equal to 2.8 minutes. Recovery for all treatments was less than 223 seconds equal to 3.7 minutes. The anesthetic should be easy to administer, effective at low doses and reasonable in cost [5, 9].

Regarding the efficacy criteria for an ideal anesthetic agent to be suitable for a researcher, it should induce anesthesia rapidly with minimum hyperactivity or stress, produce anesthesia within 3 min or less. When the animal is removed from the anesthetic and placed in recovery tank containing clean water, recovery should be rapid, within 5 min or less. Therefore, the tested clove essence concentrations are usable for doing trial operations which need the fish be anesthetized.

Although Anesthetic induction as well as recovery phase was not significantly affected by concentration, anesthetic and recovery time was respectively, decreased and increased by elevating anesthetic agent dosage.

Longer recovery time which was observed in fish anesthetized with clove essence could be an additional advantage in activities such as morphological evaluations, biopsy and stripping which are required long handling periods outside the water [14, 19, 20, 23].

Effects of water temperature were obvious in all of the concentrations. Water temperature significantly ($p<0.05$) affected on both anesthetic and recovery time. The higher the water temperature, the lower the anesthetic and recovery time.

Since environmental factors affect the efficacy of anesthetics in fish, it is not surprising that the relationship between clove essence dosage and water temperature was also significant ($p<0.05$) regarding anesthetic and recovery time.

As an ectoderm animal; body temperature of fish closely follows their environments which result in temperature-related physicochemical passage of the drug into the fish. Therefore, at lower water temperatures, higher doses or longer exposure times to anesthetic agents required due to the decrease in absorption rate [12].

Eugenol, clove active ingredients, has been widely tested for human consumption and is listed as substances generally regarded as safe (GRAS) in humans at levels less than 1500 ppm [8, 13, 14].

This suggests that the levels of clove essence used in this trial may have little or no effect on humans that consume fish treated with this anesthetic. What's more, the other major advantage of clove essence is its price and not unpleasant to work with.

Plasma cortisol as well as glucose is physiological indicators of stress in fishes and their interactive effects on metabolism during recovery from stress have recently become a subject of more intense study [28-30, 35,36, 38]. In fact, the response to environmental stress is activation of the hypothalamic-pituitary-interrenal axis with an increase in the blood plasma of the steroid hormone, cortisol [4]. Cortisol level was monitored at specific times before and after anesthetic exposure to elucidate how anesthetic solutions influence it. According to the efficacy of an ideal Anesthetic agent, it prevents increasing in cortisol level [39].

Although it is claimed that some anesthetics including clove oil blocks activation of the hypothalamo-pituitary-inter-renal (HPI) axis and releasing circulating cortisol in relation to the handling procedures [36, 40, 41], Elevating cortisol plasma upon replacement in recovery tank, as observed in our experiments, might be due to the low respiration after stage III of anesthesia which result in respiratory acidosis as well as hypoxia and consequently high blood cortisol level As was

demonstrated in fish anaesthetized with buffered TMS, 2-Phenoxyethanol, Benzocaine, Metomidate and CO2 [33, 40]. It suggests activation of HPI axis despite deeply anesthetized with clove essence. Clove oil was used to reduce stress before slaughter for comparing it's efficiently with hypothermia and asphyxia methods and similarity in plasma cortisol between unstressed and anesthetized group with this agent was observed [36]. Clove oil might act much more quickly for inducing anesthesia in senegal sole, *Solea senegalensis*, than in persian sturgeon, *Acipenser persicus*, which result in depressing of cortisol response and its normal circulation. Clove oil did not block the cortisol response to stressors operations in sea bream, *Sparus aurata*, just like happening with other anaesthetics a similar to our trial.

Hypoxia as a result of reduction in respiratory actions lead in physiological changes in the blood factors such as raising glucose and haematocrit (Hct) to combat with lowering in O2 in circulation for breathing and survival . Elevating plasma glucose as well as Hct and hemoglobin was similar to the reports for other anesthetized fish [20- 23, 42].

White Blood Cell (WBC) was measured to evaluate clove essence effect on fish immune system. It showed a decline trend associated with arresting in anesthetic in stage III. We had already observed an increase in plasma cortisol concentration which is a glucocorticoid hormone and can act as an immunosuppressive [37], so it could suppress humoral factors and lead in declining circulating WBC along with elevating cortisol.

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